GOITER STUDIES WITH THE RAT[1]

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ONE PLATE (SIX FIGURES)

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Kraus and Monroe ('30) and Levine et al. ('33) have shown that goiter in the rat is fundamentally a result of iodine deficiency. Levine and coworkers have reported that the daily iodine requirement of the rat is 1–2 μg. The present study was undertaken to determine the iodine requirement of the growing rat on a synthetic ration to which all of the known dietary essentials were added. Since soybeans are low in iodine (Wilgus, et al., '41) and contain high quality proteins (Barnes and Maack, '43), soybean globulin was selected for the protein source.

EXPERIMENTAL

Four groups of rats were used in a 13-week experiment. Each group consisted of four female white rats which weighed 39–49 gm. and averaged 43.2 gm. at the beginning of the experiment. The rats were allowed to consume the ration ad libitum. Records of food consumption and of the growth rate were kept.

The low-iodine ration (Teresi, '43) consisted of: 75 parts recrystallized sucrose, 20 parts autoclaved soybean globulin, 4 parts salts, 5 parts corn oil, 0.6 part cystine, 0.1 part choline chloride. The percentage composition of the salt mixture which was prepared from reagent grade minerals, follows: $Ca_3(PO_4)_2$, 61.0; NaCl, 20.0; KCl, 10.0; $MgSO_4 \cdot 7H_2O$, 8.0; $FeCl_2 \cdot 4H_2O$, 0.73; $CuSO_4 \cdot 5H_2O$, 0.09; $MnSO_4 \cdot H_2O$, 0.09; and $ZnSO_4$, 0.09. The $Ca_3(PO_4)_2$ was purified further by dissolving it in reagent grade HCl. After the undissolved particles had been filtered off, the $Ca_3(PO_4)_2$ was precipitated by the addition of chemically pure NH_4OH to a pH of 4. The precipitated product was filtered, rinsed with absolute alcohol, and dried at 37°C. for 24 hours.

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These vitamins were added at the following levels per kilogram of ration: 10.0 mg. calcium pantothenate, 2.0 mg. thiamine hydrochloride, 10.0 mg. riboflavin, 6.0 mg. pyridoxine, and 65.0 mg. alpha tocopherol. One hundred and twenty micrograms of beta carotene in Wesson oil and 250 U.S.P. units of crystalline vitamin D in propylene glycol (Drisdol) were fed by dropper to each rat every week.

The soybean globulin contained 80.0% protein as determined by Kjeldahl analysis. The nitrogen factor, 5.7, was used to calculate the protein content. The preparation (Teresi, '43) of the globulin involved the dialysis of an aqueous NaCl extract of raw, ground soybeans. The precipitated globulin was centrifuged with alcohol and finally with ether to purify and facilitate the drying of the final product.

The rats in group 1 received the basal ration only. Those in groups 2 to 4 received, respectively, 0.5, 1.0, and 2.0 μg. of iodine per rat per day. The iodine supplement, which consisted of potassium iodide in redistilled water was given in 3 ml. amounts contained in crock cups each morning. When the iodine supplement had been consumed, an adequate volume of redistilled water was given to supply the daily water requirements of the rats.

After 13 weeks on the experimental diet the animals were sacrificed by ether anesthesia. The thyroids were removed with the aid of a binocular dissecting microscope and weighed immediately on a torsion balance. The thyroids were then fixed in Bouin's fluid. Histological examination was made after the glands had been sectioned and stained in hematoxylin and eosin.

RESULTS

The groups supplemented with potassium iodide showed no significant differences in food consumption or rate of growth from the group which received no iodine supplement.

The thyroid glands of the rats on the basal group were definitely enlarged and red. When 0.5 μg. of iodine per rat per day was given, the thyroid enlargement was greatly reduced, but the red appearance was still noticeable. The thyroids of the groups which received 1.0 and 2.0 μg. of iodine per rat per day were smaller and more normal in color. No significant differences were noted in the gross appearance of the glands from the two groups.

Table 1 shows the rat weights at the termination of the experiment, the weights of the dissected thyroid glands, and the calculated thyroid weights per 100 gm. of rat tissue. The thyroid weights represent the

combined weights of the freshly dissected thyroid and parathyroid glands.

Upon histological examination, the thyroid glands of the rats which received only the basal ration appeared completely abnormal. Acinar epithelial hypertrophy was present in large areas of the gland and no colloid was found in the acini (fig. 1). What little colloid remained

TABLE 1

Thirteen-week experiment showing effect of iodine supplements on thyroid weights.

GROUP NO.	IODINE SUPPLEMENT PER RAT PER DAY	RAT WEIGHTS	THYROID WEIGHTS	THYROID WEIGHTS PER 100.0 GM. RAT WEIGHT
			mg.	*mg.*
I	No supplement	200.0	40.8 [1]	20.4
		188.0	38.4 [1]	20.4
		235.0	67.1 [2]	28.6
		180.0	24.6 [1]	13.7
Average				20.8
II	0.5 μg. iodine	179.0	20.4 [2]	11.4
		200.0	24.0 [2]	12.0
		193.0	25.0 [1]	13.0
		206.0	27.8 [1]	13.5
Average				12.5
III	1.0 μg. iodine	201.0	19.0	9.5
		181.0	18.8	10.4
		130.0	15.0	11.5
		176.0	16.5	9.4
Average				10.2
IV	2.0 μg. iodine	191.0	19.2	10.1
		209.0	14.1	6.8
		194.0	24.1 [2]	12.4
		129.0	14.8	11.5
Average				10.2

[1] Gland of red appearance.
[2] Gland of pink appearance.

contained large numbers of necrotic nuclei (fig. 2). In other areas, a necrosis of the acinar epithelium had occurred which varied from a mild involvement (fig. 3), to a complete necrosis (fig. 4) and a disappearance of any semblance of normal structure or arrangement (fig. 5). There were fewer areas of complete necrosis in the thyroids of the rats which received 0.5 μg. of iodine per day; otherwise, they resembled the thyroids of the basal group. No normal areas were observed. The thyroid

glands of the rats which received 1.0 μg. of iodine per day showed widespread areas of early necrosis and hypertrophy with no accumulation of colloid. A few small areas of fairly normal acinar epithelium with colloid accumulation were found. However, in all cases, numerous necrotic nuclei were present in the colloid. The thyroid glands of those rats which received 2.0 μg. of iodine per day were almost normal (fig. 6). There were large areas of normal acini with distinctly flattened, cuboidal epithelium and smooth vacuolated colloid; however, a few small areas with necrotic nuclei in the colloid and mild necrosis of the epithelium were observed.

DISCUSSION

The results indicate that iodine is the determining factor in preventing the development of goiter in rats on this ration. The maximum weekly growth observed was 24 gm. per rat during the fifth week of the experiment; however, the overall rate of growth was not normal.

Levine et al. ('33) obtained weekly gains of 10 to 12 gm. during 5-week experiments in which a modified Steenbock rachitic ration was fed. This ration contained 14–17 parts of iodine per billion and furnished an average of 0.15 μg. per rat per day. These workers were able to prevent goiter in rats on this ration by adding iodine as potassium iodide to furnish a daily supply of 1 to 2 μg. per rat.

Remington ('37) incorporated dried pig liver into a modification of the Steenbock rachitic diet. Young rats grew 20 gm. per week on this ration. Rats reared on this ration grew to maturity and produced normal numbers of living young. In spite of the fact that the 2.0% liver contributed 0.04 μg. of iodine per day per rat, the thyroid enlargement was 3.7 times the normal as compared to 2.4 times the normal when no liver was included in the ration. It was found that 2.0% dried brewer's yeast gave the same growth increase as 1.0% dried pig liver in the modified Steenbock rachitic ration. The addition of yeast, which contained an insignificant amount of iodine, resulted in increased growth without affecting the degree of thyroid enlargement; hence, the increased thyroid hyperplasia caused by various levels of pig liver cannot be attributed to the increased rate of growth. Remington explains the goitrogenic effect of liver as being caused by the presence in liver of a substance the metabolism of which causes a drain on the thyroid mechanisms or of a specific thyroid stimulating hormone. Remington and Remington ('38) were able to reduce the hyperplastic thyroids of rats reared on the 2.0% pig liver ration to near normal by feeding 5–6 μg. of iodine as sodium iodide daily.

The type of ration consumed, apparently, has a marked effect on the rat's iodine requirement. In the present experiment, the thyroids of the rats receiving the 1.0 and 2.0 μg. supplements had an average weight of 10.2 mg. per 100 gm. of rat weight as compared to 8.8 ± 0.4 mg. per 100 gm. which, according to Remington, is the normal thyroid weight for 185-gm. rats.

Histological studies showed that 1 and 2 μg. supplements did not entirely correct the thyroid pathology. The pathological changes observed in the thyroid glands of the rats which received the basal ration only were much more extensive than has been previously reported by Hellwig ('32, '35), Thompson ('32), and Remington and Remington ('38). The possibility that soybean globulin contains a substance which causes an excessive strain on the thyroid mechanism either through increased metabolic demands or through direct stimulation cannot be ignored. However such a substance can hardly be the goitrogenic factor reported by Wilgus et al. ('41) which they found to be destroyed by heat, because the soybean protein we used was autoclaved. With respect to the Sharpless ('38) report that this goitrogenic substance can be extracted with fat solvents, a finding that Wilgus and associates were unable to confirm, it should be pointed out that the soybean protein we used was free from fat.

SUMMARY

A ration is reported which will produce an iodine deficiency in the white rat. Definite enlargement, redness, and histological changes were noted in the thyroid glands of the rats on the basal ration and on the basal plus 0.5 μg. of iodine per rat per day. The addition of 1.0 and 2.0 μg. of iodine per day to the basal ration largely corrected the glandular enlargement. The pathological changes in the thyroids were partially prevented by 1.0 μg. of iodine per day; however, 2.0 μg. resulted in almost complete prevention of pathological changes. No differences in growth or food consumption were noted between the basal group and the groups on the iodine supplements.

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PLATE 1

EXPLANATION OF FIGURES

HISTOLOGICAL DESCRIPTIONS

1 A section of the thyroid gland of a rat which received the basal ration only. Epithelial hypertrophy. H and E. × 440.

2 A section of the thyroid gland of a rat which received the basal ration plus 1.0 μg. of iodine per day. Epithelial hypertrophy and necrotic nuclei in the acinar lumina. H and E. × 440.

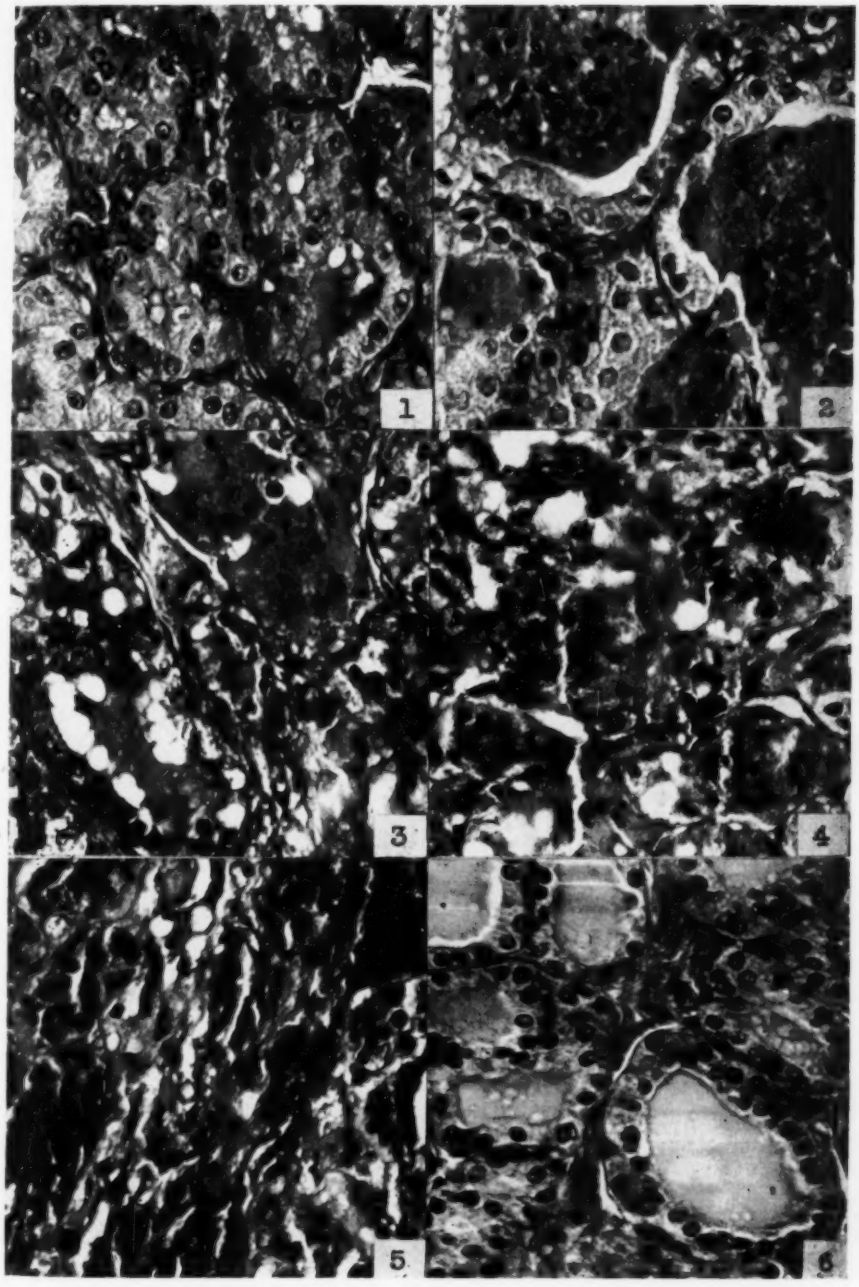
3 A section of the thyroid gland of a rat which received the basal ration only. Beginning of epithelial necrosis. H and E. × 440.

4 A section of the thyroid gland of a rat which received the basal ration only. More extensive epithelial necrosis. H and E. × 440.

5 A section of the thyroid gland of a rat which received the basal ration only. Advanced and widespread necrosis. H and E. × 440.

6 A section of the thyroid gland of a rat which received the basal ration plus 2.0 μg. of iodine per day. Normal. H and E. × 440.

PLATE 1



PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

I. THE HUMAN BIOASSAY TECHNIC [1]

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ONE FIGURE

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Among the urgent needs for further research reported at the National Nutrition Conference in 1941 was "Improvement of presently known chemical and biological procedures for estimating the amounts of the essential nutrients in foods and their physiological availability." In this statement there are recognized not only the advantages of chemical and microbiological assays for vitamins, a field of methodology in which outstanding progress has been effected, but also the basic principle that content and availability are not synonymous terms in the case of vitamins any more than they are with respect to minerals, proteins, or carbohydrates. Analytical methods are directed toward establishing the total quantity of a specific nutrient present in a food. On the other hand, that fraction of the total nutrient which is available for absorption is measured by biological tests preferably on the animal species for which the food is intended. The need for further studies along these lines has recently been emphasized (Hart, '45).

Vitamins may exist in nature in such firm union with proteins or other compounds that they either fail to react chemically or are not utilized by the test microorganism until released by preliminary hydrolysis. This pretreatment of the test sample may not be duplicated by the digestive processes during the gastrointestinal journey.

Animal assays are usually regarded as measuring physiologically available nutrients, but they are not always reliable when applied to human nutrition. Among the factors affecting animal assays are the need for subdivision or extraction of the test materials, the pathologically depleted state of the experimental animal, the use of purified or

[1] This paper is one in a series of reports dealing with the physiological availability of the vitamins. It was presented in part before the Division of Biological Chemistry at the 108th meeting of the American Chemical Society, New York, N. Y. The expenses of this study were defrayed by a grant from Lever Brothers Company, Cambridge, Massachusetts.

unbalanced basal rations whose composition may be specifically related to the utilization of the factor under test; these depart considerably from the conditions underlying the nutrition of man, and therefore warrant the exercise of caution in the application of laboratory data.

The present report describes the procedure for the assay of physiologically available vitamins, or for the study of conditions affecting their availability, directly on human subjects. The technic permits the simultaneous assay of the various water-soluble vitamins in foods or pharmaceutical products in the condition in which they are normally consumed. Since the test materials are fed in a single dose, it is possible to assay perishable materials without making assumptions as to stability such as are often necessary in prolonged animal assays. Evaluation of the rate as well as degree of availability can be made by this procedure. In cases where repetition of the control period may be dispensed with, the entire assay can be completed within 1 week. The precision of the human bioassay is at least as good as that of most animal assays.

Using normal subjects as the basis for comparison, it is possible by this technic to determine the effect of various conditioning factors (Jolliffe, '43), both physiological and pathological, on the availability of the water-soluble vitamins. Since the procedure in its present form is based on a standardized urinary response, its application is limited to those vitamins normally excreted via the renal route, namely, the water-soluble vitamins. A number of studies demonstrating the versatility and the reliability of the new assay technic have been completed and are being presented for publication.

EXPERIMENTAL PART

Principle of bioassay technic. In normal human subjects the urinary excretion of the water-soluble vitamins, as such or as their derivatives, is directly proportional to the quantity consumed, provided that at the time of the tests the subjects are subsisting on an adequate diet. The linear dose-response relationship established by feeding the vitamins in pure solution, i.e., in their most completely available form, constitutes the basis of the bioassay for the estimation of available ascorbic acid, thiamine, riboflavin and nicotinamide.

Collection of urine samples and analytical methods. The 24-hour urine samples are collected in opaque bottles containing 20 ml. of 3.5 N sulfuric acid and stored at room temperature. The ascorbic acid analyses are conducted immediately after completion of the samples. The

thiamine, riboflavin and N^1-methylnicotinamide in these samples are stable for a period of at least 2 weeks.

Ascorbic acid may be found in the urine as such and as the partially oxidized form, dehydroascorbic acid. The conversion of reduced to dehydroascorbic acid occurs not only in vivo (Berryman, French, Harper and Pollock, '44) but also during storage of the urine samples. It is markedly inhibited by low pH. The photometric procedure of Bessey ('38) as modified by Hochberg, Melnick and Oser ('43) has been used to determine both forms of urinary ascorbic acid. In this and subsequent reports only values for total ascorbic acid are reported. In general, about 90% of the total excreted vitamin is found in the reduced state, the remainder being dehydroascorbic acid.

Thiamine is excreted in the urine as the free vitamin (Melnick and Field, '39). It has been estimated by the simplified colorimetric method (Hochberg and Melnick, '44). Riboflavin has been determined by a modification (Strong and Carpenter, '42) of the microbiological method of Snell and Strong ('39) and by the fluorometric procedure described by Najjar ('41) omitting the blank correction for non-riboflavin fluorescent compounds. The microbiological procedure is more specific for riboflavin in urine than the abbreviated fluorometric procedure. However, the latter is far more precise and since conclusions are based upon the extra urinary excretion of the vitamin following riboflavin dosage, the calculations themselves correct for the presence of the irrelevant fluorescent materials in the two consecutive urine samples. It has also been found that the extra urinary excretions of riboflavin estimated by microbiological and fluorometric analyses give the same average values, but the figures for the individual test subjects vary more widely when the microbiological method is employed. N^1-Methylnicotinamide, the main excretory product following nicotinamide ingestion (Najjar and Wood, '40; Huff and Perlzweig, '43) has been determined fluorometrically (Hochberg, Melnick and Oser, '45).

Basal diet. The composition of the basal ration used in this and subsequent studies is shown in table 1. Analyses were conducted on aliquots of a composite sample of the diet. All foods containing active enzymes were first blanched under an atmosphere of nitrogen and the cooked foods, together with the original blanching fluids, were homogenized likewise under nitrogen. Aliquots were then promptly taken for the tests. Proximate analyses were conducted by the methods of the Association of Official Agricultural Chemists ('40). The same photometric procedure employed for the urine analyses was used for the determination of the ascorbic acid content of the diet. Riboflavin was

estimated by the microbiological procedure (Snell and Strong, '39); thiamine by the thiochrome method (Hennessy, '41); and nicotinic acid colorimetrically as described by Melnick ('42).

The content of the essential nutrients, listed in table 1, indicates that the basal diet furnishes sufficient protein of excellent quality and considerably greater quantities of the water-soluble vitamins than are

TABLE 1

Composition of basal diet.

	Breakfast		
1 orange	150 gm.	2 butter squares	14 gm.
2 slices of toast (enriched)	50 gm.	1 glass milk	230 gm.
	Dinner		
1 steak (lean)	150 gm.	5 butter squares	35 gm.
1 serving of fried potatoes	65 gm.	1 glass of milk	230 gm.
1 serving of carrots	60 gm.	1 serving of apple pie	155 gm.
1 serving of beets	70 gm.	2 hard sugar candies	12 gm.
2 slices of toast (enriched)	50 gm.		
	Supper		
2 fried eggs	90 gm.	1 glass of milk	230 gm.
1 lettuce serving	25 gm.	1 apple	150 gm.
1 tomato serving	70 gm.	1 banana	150 gm.
2 slices of bread (enriched)	66 gm.	2 hard sugar candies	12 gm.
4 butter squares	28 gm.		

Analyses conducted on aliquots of the composite diet.

Proximate analysis	*Values found*[1]	*Vitamin content*	*Values found*[1]
Total weight	2075 gm.	Thiamine	1.34 mg.
Total solids	540 gm.	Thiamine: Calorie ratio	0.5
Moisture	1535 gm.	Thiamine: Non-fat calorie ratio	0.9
Protein	101 gm.	Ascorbic acid	115 mg.
Fat (ether extract)	129 gm.	Riboflavin	2.54 mg.
Ash	22 gm.	Nicotinic acid[2]	22.0 mg.
Crude fiber	6 gm.		
Carbohydrate (by difference)	282 gm.		
Caloric value	2710 Cal.		
Non-fat calories	1550 Cal.		

[1] Expressed in terms of total food consumed in the 3 meals.

[2] Predominantly as the amide.

generally regarded as minimal for adequate nutrition. The thiamine: calorie ratio (Cowgill, '34) and the thiamine: non-fat calorie ratio (Williams and Spies, '38) are adequate for proper nutrition.

Selection of test subjects. For the availability studies it is desirable to use at least five subjects, preferably males. Inasmuch as deficient subjects retain extra dietary vitamin, a reliable ratio of extra urinary

excretion indicative of availability can be obtained only with subjects whose nutritional status is normal. Dietary histories obtained from daily records of food consumption kept by the prospective subjects yield first approximations on the suitability of the individuals for the study. However, such an approach is known to be of limited value, since dietary histories may very often be untrustworthy. For this reason it is essential to rely primarily on objective vitamin clearance tests for the selection of the subjects. Those employed for the present availability studies subsisted regularly on diets furnishing nutrients in amounts comparable to those listed in table 1 for the basal diet and excreted the vitamins well within the normal range both before and after postprandial dosage with extra vitamins. Typical urinary excretion values obtained with these subjects are given in table 2.

TABLE 2

Urinary excretion of vitamins (or derivatives) by test subjects.
(All values in milligrams.)

SUBJECT	SURFACE AREA IN SQ. M.	EXCRETION [1] OF VITAMIN OR DERIVATIVE, PER 24 HRS.							
		Thiamine		Ascorbic acid		Riboflavin		N^1-methylnicotinamide [3]	
		Basal	Increment after test dose [2]	Basal	Increment after test dose [2]	Basal	Increment after test dose [2]	Basal	Increment after test dose [2]
J.C.	1.70	0.33	1.03	18	121	0.73	4.26	8.0	14.7
E.M.	1.76	0.42	0.62	18	101	0.78	4.50	7.2	13.6
D.M.	1.88	0.39	0.93	36	117	0.57	4.84	7.7	9.8
M.H.	1.96	0.27	1.04	60	113	0.71	4.65	4.8	5.2
H.H.	2.15	0.31	0.84	52	121	0.74	4.70	3.8	7.7
Average	1.89	0.34	0.89	37	115	0.71	4.59	6.3	10.2

[1] On the day that urine samples were collected, each subject consumed the diet shown in table 1.

[2] The test dose of 5 mg. thiamine, 200 mg. ascorbic acid, 10 mg. riboflavin and 50 mg. nicotinamide in pure solution was taken orally immediately after dinner.

[3] Results expressed as nicotinamide on an equimolar basis.

Description and experimental justification of the assay technic. In biological assays with laboratory animals the same standardized diet is ingested daily throughout the period of observation. Such a technic cannot be applied readily to humans employed for bioassay purposes. For this reason marked simplification of the feeding routine is necessary. It has been found sufficient to have the test subjects consume the basal diet (see table 1) only on the days that urinary collections are made, allowing them to return to their customary but still adequate diets during the intervening periods. Under such circumstances monotony in the dietary is avoided and the routine feeding so simplified

that no difficulties are encountered in obtaining full cooperation from the test subjects.

Details of the method for conducting biological assays with human subjects can best be described by reference to a simple example. The problem was to determine the availability of the water-soluble vitamins in a pharmaceutical vitamin tablet when the product is swallowed whole.[2] Obviously disintegration in vivo is a necessary preliminary to absorption. The rat cannot be relied upon to measure availability since the intact tablet is too large to be administered. Bioassays with even larger laboratory animals would also be unsatisfactory since these would involve the feeding of curative or prophylactic doses of the vitamins in amounts far less than those contained in a whole tablet.

As an example of the data obtained in determining the availability of a vitamin, a test for ascorbic acid is summarized in table 3. Five

TABLE 3

Availability to man of ascorbic acid in a multivitamin-mineral tablet.

SUBJECT	CONTROL PERIOD: PURE SOLUTION DOSE, 200 MG. ASCORBIC ACID			TEST PERIOD: 15 TABLET DOSE 173 MG. ASCORBIC ACID		
	Basal excretion	After test dose	Test dose excreted	Basal excretion	After test dose	Test dose excreted
	mg./24 hours		%	*mg./24 hours*		%
J.C.	57	183	63	35	116	47
E.M.	41	159	59	18	92	43
D.M.	47	131	42	38	128	52
M.H.	70	162	46	17	96	46
H.H.	28	93	33	20	109	52
Average	49	146	48.6	26	108	48.0

Availability of ascorbic acid in tablets $= \frac{48.0}{48.6} \times 100 = 98.8 \pm 11.9\%$.

test subjects were employed for the assay. The basal urinary excretions of the vitamin on the planned adequate diet (table 1) were measured. Immediately after the mid-day meal, the subjects were given an aqueous solution of ascorbic acid [3] and the extra urinary excretion of

[2] The War Food Administration includes in certain contract specifications the proviso that the vitamins must be present not only in amounts to meet label claims, but that these factors must also be available when tablets or capsules are swallowed intact (Federal Surplus Commodities Corporation, '44).

[3] Actually the pure solution dose of ascorbic acid also contained the other water-soluble vitamins for which assays were being made. Studies have indicated that the extra urinary excretion of the water-soluble vitamins (or derivatives), thiamine, ascorbic acid, riboflavin and N^1-methylnicotinamide, by the normal subject subsisting on the basal diet are the same regardless of whether the vitamin is taken alone or in various combinations. It is important that the test doses of extra vitamins be taken postprandially, as indicated, or non-reproducible excretion values may be obtained; variations in the stability of some vitamins and in the rates and degrees of absorption of others are noted at different stages of gastrointestinal function.

the vitamin measured. The proportion of the test dose found in the 24-hour urine sample was then calculated; in this case it was 49% of the 200 mg. extra ascorbic acid taken. A 2 weeks' period was allowed to elapse during which the subjects returned to their original state of nutrition. The procedure was then repeated, but in place of the pure solution of the vitamin, the test material, containing a comparable quantity of ascorbic acid, was taken.[4] In this example, fifteen tablets were swallowed whole. The extra urinary excretion was then measured and the per cent of the test dose found in the urine was calculated. In the assay described extra quantities of ascorbic acid following tablet dosage could appear in the urine only after extraction of the vitamin from the disintegrated tablet and absorption from the gastrointestinal tract. Since the mean percentage of the test dose excreted after ingestion of the tablets equaled that when the vitamin was administered in the control solution, it was concluded that the ascorbic acid in the vitamin tablets was completely available.

The individual data for the test subjects in the ascorbic acid assay were actually not as reproducible from control to test period as the data for the other water-soluble vitamins, particularly with respect to the basal excretion values. However, it was precisely this greater variability in the ascorbic acid assay which led us to select it for detailed presentation because it demonstrates that such variations in the basal excretion values introduce no error in the assay. Nevertheless it is worthy of note that even in this example the standard error of the ratio of excretion in the two periods was only about 12% which is less than the error of most animal assays.

At first glance the discrepancies in the urinary excretion responses appear to be large. For example, M. H. excreted a total of 162 mg. of ascorbic acid during the control period but only 96 mg. during the test period. However, the basal value for the subject during the control period was markedly greater than that during the subsequent test period so that the same percentages of the test dose were excreted in the urines. Inasmuch as the basal figure may show this large variation from one period to another, the question may be raised — What justification is there for subtracting the basal urinary excretion from the total value to arrive at an amount attributable to the extra dosage? Data illustrating the reproducibility of the basal excretion on consecu-

[4] If the object is to study some conditioning factor which might interfere with the absorption of the vitamin, the extra vitamins are also taken during the test period in pure solution but with the imposition of the conditioning factor. Other modifications in the assay technic are frequently necessary as illustrated in forthcoming publications.

tive days are shown in table 4. Daily basal excretion values were obtained for the five subjects during different periods of 2 and 3 days, respectively. It will be noted that M. H. excreted far less ascorbic acid during period A than during period B but that the basal figures are reproducible within each period. Similar results were obtained with all of the subjects with respect to basal excretion values not only

TABLE 4

Reproducibility of basal urinary excretion values on consecutive days.

(All values in milligrams per 24 hours.)

FACTOR EXCRETED	SUBJECT	PERIOD A			PERIOD B			
		1st day	2nd day	Average	1st day	2nd day	3rd day	Average
Ascorbic acid	J.C.	36	45	41	36	45	57	46
	E.M.	17	12	15	74	61	59	65
	D.M.	56	38	47	40	44	43	42
	M.H.	18	17	18	67	59	57	61
	H.H.	29	20	25	22	23	27	24
	Average	31	26	29	48	46	49	48
Thiamine	J.C.	0.35	0.33	0.34	0.22	0.23	0.23	0.23
	E.M.	0.45	0.42	0.44	0.25	0.19	0.19	0.21
	D.M.	0.37	0.39	0.38	0.25	0.29	0.28	0.27
	M.H.	0.30	0.27	0.29	0.19	0.24	0.22	0.22
	H.H.	0.29	0.31	0.30	0.32	0.28	0.26	0.29
	Average	0.35	0.34	0.35	0.25	0.25	0.24	0.25
Riboflavin	J.C.	0.64	0.74	0.69				
	E.M.	0.85	0.95	0.90				
	D.M.	0.65	0.69	0.67				
	M.H.	0.75	0.84	0.80				
	H.H.	0.63	0.68	0.66				
	Average	0.71	0.78	0.74				
N^1-Methylnicotinamide (as nicotinamide)	J.C.	7.8	6.2	7.0				
	E.M.	6.7	5.9	6.3				
	D.M.	6.7	6.7	6.7				
	M.H.	3.8	4.5	4.2				
	H.H.	3.7	3.0	3.4				
	Average	5.8	5.2	5.5				

for ascorbic acid but for thiamine, riboflavin and N^1-methylnicotinamide as well. In any event the small daily variations within a given period are inappreciable in terms of the extra excretions following dosage. These findings justify the subtraction of the basal value on 1 day from the total excretion on the subsequent day to arrive at the quantity of extra urinary vitamin (or derivative) directly related to dosage.

In order to employ the ratio of the extra urinary excretion of the vitamin during the test period to that during the control period as an index of availability, it is necessary to demonstrate that a reproducible linear relationship exists between dosage and response. Figure 1 illustrates that, with increasing intake of any one of the four water-soluble vitamins studied, there is an increase in its extra urinary excretion. Good agreement is obtained among the test subjects receiving the three test doses of ascorbic acid; only one value for one subject

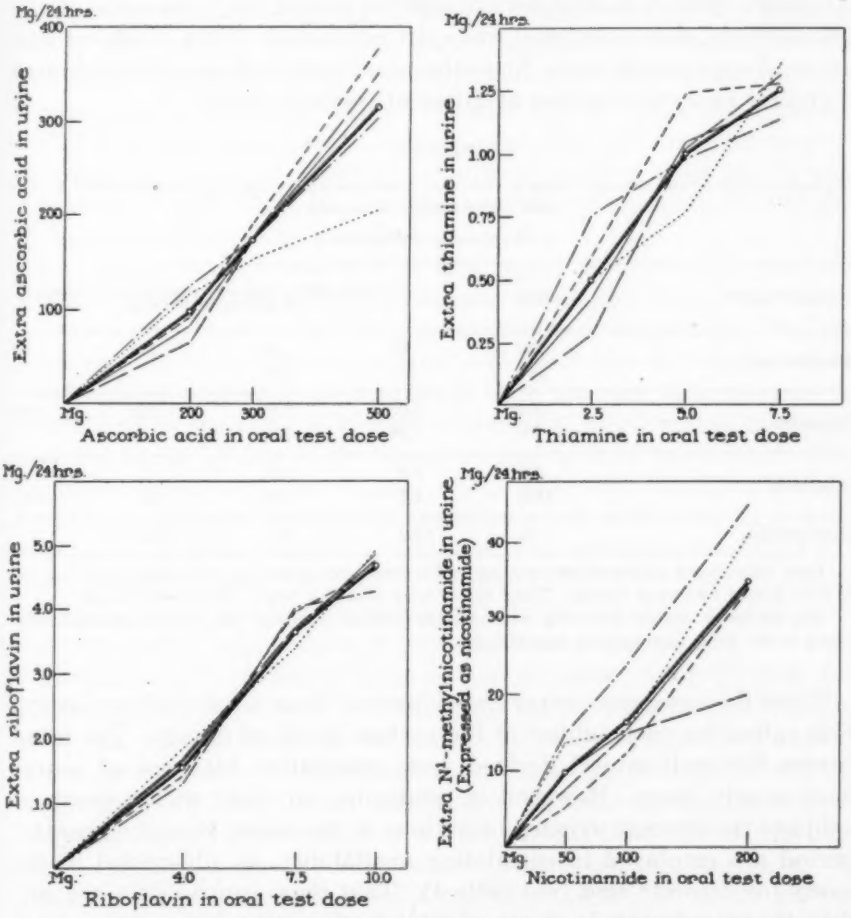

Fig. 1 The linear relationship between dosage with the water-soluble vitamins, ascorbic acid, thiamine, riboflavin and nicotinamide, and the extra urinary excretion of the vitamins (or derivative). The test doses in aqueous solution (pH 3.0) were taken orally immediately after dinner. The fine lines represent the responses of the individual subjects whereas the heavy line indicates the average response.

showed an appreciable deviation from all the others. In the case of thiamine the uniformity of linear response among the various subjects was not quite as good. However, in every case with increasing dosage there was an increment in the urinary output of the vitamin so that the average curve is linear. The dose-response relationship in the case of riboflavin is excellent, the values obtained with the five subjects being in very close agreement. The graph for the extra urinary excretion of N^1-methylnicotinamide following nicotinamide dosage is of interest. Although there was considerable spread among the responses of the test subjects, each individual was quite consistent. Thus, a subject who excreted appreciably more N^1-methylnicotinamide than another showed a greater urinary excretion at different levels of intake.

TABLE 5

Reproducibitily of the average urinary excretion responses following oral post-prandial dosage with water-soluble vitamins.[1]

(All values in milligrams.)

VITAMIN TAKEN	DOSE	AVERAGE EXTRA URINARY EXCRETION OF VITAMINS AT DIFFERENT PERIODS			
Ascorbic acid	200	97	94	108	93
	300	173	150		
Thiamine	5.0	1.02	0.90	1.09	0.92
	7.5	1.26	1.26		
Riboflavin	7.5	3.8	3.6		
	10.0	4.7	4.9	5.6	5.3
Nicotinamide[2]	50	10.2	8.5	9.4	

[1] Each value listed represents the average of the responses by the five test subjects during the 24-hour period following dosage. These values were obtained over a period of 2 years.

[2] The excretion values following nicotinamide dosage represent the N^1-methylnicotinamide found in the urine expressed as nicotinamide.

These dose-response curves were plotted from single urinary excretion values for each subject at the various levels of dosage. The mean curves for each subject derived from cumulative data are of course more nearly linear. However, in conducting an assay with a group of subjects the average urinary excretions in the single preceding control period are employed in calculating availability, as illustrated in the assay for ascorbic acid (see table 4). That these control data are sufficiently reproducible is shown in table 5, giving the average responses for the same five subjects taking different quantities of the vitamins at widely spaced periods. Only during the urinary collection periods

were the subjects fed the basal diet (see table 1); at other times they subsisted on their regular adequate dietaries.

DISCUSSION

Measurement of the urinary excretion of the water-soluble vitamins as a means for the early diagnosis of avitaminosis has been repeatedly criticized on the basis that it reflects only the immediate vitamin intake. This, however, is true only in the case of normal subjects since deficient individuals retain extra dietary vitamin to replenish depleted stores. This prompt response of normal subjects to variations in vitamin intake is utilized in the procedure here described for determining physiological availability. Since the water-soluble vitamins are not excreted to a significant extent in the feces, when administered as pure solutions in the doses selected, the present method does not involve analysis of stools. Thus, it is possible to avoid complications introduced by bacterial synthesis in the colon.

The assay technic described in this report is a simplification and standardization of procedures previously employed. Using the values for the urinary excretion of thiamine as indices of availability, Melnick, Robinson and Field ('41) were able to demonstrate that the increased requirements of peptic ulcer patients were not due to a decrease in availability of the vitamin because of the high fat content of the Sippy diet, but to the effects of the accompanying antacid medication. Subsequently Parsons and associates ('42 a, '42 b, '44) demonstrated also by urinary excretion studies that the vitamins of the B complex in fresh yeast were available only to a limited extent, and more recently (Williamson and Parsons, '45) that an increase in the crude fiber content of the ration did not reduce thiamine availability. In those as well as in our own studies, each subject served as his own control.

In studies with various pharmaceutical products (Oser, Melnick and Hochberg, '45) it has been found that the technic permits simultaneous assays for several vitamins and tests to be conducted on the materials in the physical state in which they are taken by the human, i.e., without requiring prior extraction or subdivision. By determining the urinary excretion values for consecutive 4-hour periods following dosage, the rate as well as degree of vitamin availability can be determined. In assays of standardized products it has been found that the values following pure solution dosage are so reproducible that repetition of the control series each time is unnecessary. Assays may then be completed within a week.

Further evidence for the reliability of the simplified assay technic described in this report has accumulated in studies, now in press, which involved application of the procedure to problems in human nutrition. These include investigations on the influence of the ingestion of live yeast, enzymes, certain ions, or adsorbents on the availability of the vitamins.

Because of their instability certain products, when subjected to rat assay, must be stored under conditions which prevent spoilage or be replaced by fresh samples at frequent intervals in the course of the assay. The present assay procedure, however, is of such short duration that no assumptions need be made as to the stability or reproducibility of the material from batch to batch.

SUMMARY

A biological assay technic has been described for the simultaneous determination of the physiological availability of the water-soluble vitamins, ascorbic acid, thiamine, riboflavin and nicotinamide. The test is based upon the observation that the urinary excretion of the water-soluble vitamins, as such or as their derivatives, parallels the quantity consumed provided that normal test subjects are employed and that the subjects at the time of the tests are subsisting on an adequate diet. A simplified feeding routine is described which extends the applicability of the assay to quantitative studies of problems in human nutrition, particularly those dealing with the factors which contribute to conditioned malnutrition. The dose-response relationships for each of the test vitamins have been found to be linear and the reproducibility of the data superior to that encountered in most animal assays.

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PHYSIOLOGICAL AVAILABILITY OF THE VITAMINS

II. THE EFFECT OF DIETARY THIAMINASE IN FISH PRODUCTS [1]

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Chastek paralysis, an acute dietary disease of foxes, is caused by including 10% or more of certain species of uncooked fish in the diet and may be prevented or cured by giving adequate amounts of thiamine (Green and associates, '42). The enzymic nature of the anti-thiamine factor is indicated by its heat-lability (Sealock and collaborators, '43), by its reactions in the presence of enzyme inhibitors (Sealock and Goodland, '44), and by the isolation of the pyrimidine and thiazole derivatives following the hydrolytic cleavage of the vitamin (Krampitz and Woolley, '44).

On the basis of the study conducted by Deutsch and Hasler ('43) it has been generally concluded that the anti-thiamine factor is found only in fresh-water fish. These investigators reported the presence of this factor in fifteen out of thirty-one species of fresh-water fish but in not one of nine species of salt-water fish. Others, however, have found it in the Atlantic herring and whiting and in the Pacific mackerel. In addition, thiamine deficiency has been observed in cats fed a diet consisting exclusively of salt-water herring (Smith and Proutt, '44).

In discussing the subject of thiamine inactivation by raw fish, Nutrition Reviews ('43) states, "This subject may not appear to have a direct bearing on human nutrition since it is unlikely that many people are fond of eating uncooked fish." However, there are a number of popular fish products which do not undergo excessive heat processing and are intended for direct human consumption. Woolley ('43) has reported the presence of a substance in clams capable of destroying thiamine. Clams are consumed in the raw state as are also oysters, herring, and caviar. Smoked fish may be processed at as low temperatures as 27° to 65°C.

[1] Some of the results in this paper were presented in summary before the Division of Biological Chemistry at the 108th meeting of the American Chemical Society, New York, N. Y. The expenses of these studies were defrayed by a grant from Lever Brothers Company, Cambridge, Mass.

In the present study an investigation was made of the presence of the thiaminase in various fish (including shell-fish) products and the influence of this factor on the physiological availability of dietary and supplementary thiamine.

EXPERIMENTAL PART

In table 1 are presented the results of tests to determine the thiaminase activity of a variety of fish products intended for direct human

TABLE 1

Thiaminase activity of fish products intended for direct human consumption.

PRODUCT	pH [1]	THIAMINE RECOVERED FROM SUSPENSION	
		Untreated material [2]	Heated material [3]
		%	%
Clams			
Chowder	7.0	3	89
Steamer	6.9	1	92
Cherrystone	7.1	0	95
Herring			
Salted	6.3	40	89
Marinated (brand A)	4.6	48	96
Marinated (brand B)	4.5	87	103
Oysters	6.6	102	96
Smoked carp	6.7	96	99
Smoked salmon	6.2	98	101
Salmon caviar	6.0	100	100

[1] Of homogenized aqueous suspension.

[2] 10-gm. samples of each ground product were suspended at their natural pH in 40 ml. of water containing 100 μg. of added thiamine, and incubated 6 hrs. at 37°C.

[3] 10 gm. of each of the homogenized samples were heated for 20 minutes in 10 ml. of boiling water at pH 4.5 to inactivate enzymes. To the cooled suspension, diluted to 40 ml. and adjusted to the initial pH, were added 100 μg. of thiamine.

consumption. Ten-gram samples of each product were suspended in 40 ml. of water and the mixture homogenized. One hundred micrograms of thiamine were then added and the suspension incubated for 6 hours at 37°C. The control series consisted of suspensions heated [2] to inactivate the enzymes.

The results, presented in table 1, indicate that homogenized clams are very effective in destroying thiamine and that the agent responsible

[2] Each suspension was heated for a period of 20 minutes at 100°C. and pH 4.5, then adjusted to its initial pH value and finally cooled to 37°C. Destruction of thiamine in the suspension of the untreated fish product over and above that occurring in the corresponding heated sample may be attributed to the presence of the thiaminase.

for the destruction of added vitamin is heat labile. Complete destruction of thiamine was obtained with all three varieties of clam tested, namely, chowder, cherrystone and steamer. Herring, a salt-water fish, also contained the enzyme but probably in smaller concentration. Only 60% destruction of the added thiamine was noted in a case of the raw product and variable losses with the marinated preparations. No evidence was found for the presence of thiaminase in oysters, salmon caviar, smoked carp and smoked salmon; theoretical recoveries of the added thiamine were obtained in the suspensions of untreated homogenized samples. Apparently smoke-processing of the carp destroys the thiaminase originally present in the tissues of this species.

On the basis of the results obtained with the various fish products, it was decided to limit the human availability study to an investigation of the effectiveness of the thiaminase derived from chowder clams. Additional in vitro experiments have also been conducted. The test subjects were the same as those employed in the previous study (Melnick, Hochberg and Oser, '45). The only major deviation in the composition of the basal ration from that routinely employed was inclusion of clams at each meal. The total quantity of clams consumed during the day, 100 gm., is usually regarded as an "average serving". The purpose in partitioning this serving among the three meals of the day was to allow a more measurable destruction of the thiamine in vivo, if such should be the case, since the clams would always accompany the dietary thiamine. Thiamine intake was determined by the thiochrome method (Hennessy, '41) and urinary excretion by the colorimetric procedures [3] (Melnick and Field, '39; Hochberg and Melnick, '44). In the earlier report (cited above) the rationale and experimental support for the human availability technic were discussed.

In vitro tests. In table 2 are presented the results of in vitro experiments on the activity of the thiaminase derived from clams. Two series of tests were conducted, both on the homogenized basal diet. For one series, the homogenized clams were first heated [4] in order to inactivate all enzymes present. For the other, the unheated ground clams were added to the homogenized ration. The dietary mixtures in open beakers

[3] The simpler colorimetric method (Hochberg and Melnick, '44) is preferred for measurements of the urinary thiamine when appreciable amounts are present. However, because conclusions were to be drawn in this study not only from the extra urinary thiamine following test dosage but also from the lower basal excretion values, the original colorimetric procedure (Melnick and Field, '39) was also employed.

[4] One hundred grams of the ground fresh clams were heated for 20 minutes in 100 ml. of boiling water at pH 4.5 to inactivate the thiaminase. The mixture was then adjusted to its initial pH of 7.0 and added to the homogenized diet.

were then incubated for 6 hours at 37°C. at their natural pH of 5.5 and gently stirred every half hour.

The results summarized in table 2 indicate that during the incubation of a homogenized ration containing the heated clam mixture, a loss of only 15% of the thiamine occurred. A comparable percentage loss was noted in the case of the added thiamine.

The addition of the raw clam suspension to the dietary mixture resulted in marked destruction of the thiamine. This occurred during the first 15 to 20 minutes required for the preparation of a uniform mixture for sampling. Subsequent incubation of this dietary mixture failed to reduce any further the value for residual thiamine. The addition of 7.5 mg. of thiamine to the homogenized basal diet containing the raw

TABLE 2

In vitro tests of the activity of thiaminase in clams.

EXPERIMENT No.	Conditions[1]	TOTAL THIAMINE FOUND IN DIET
		mg.
I	Homogenized basal diet containing heated[2] clams	1.34
II	1 incubated 6 hrs. at 37°C.	1.14
III	1 + 7.5 mg. of thiamine	8.30
IV	III incubated 6 hrs. at 37°C.	7.60
V	Homogenized basal diet containing raw clams	0.21
VI	V incubated 6 hrs. at 37°C.	0.21
VII	V + 7.5 mg. of thiamine	0.20
VIII	VII incubated 6 hrs. at 37°C.	0.20

[1] All homogenized mixtures were incubated at pH 5.5.

[2] 100 gm. of ground clams (flesh) were heated for 20 minutes in 100 ml. of boiling water at pH 4.5. The mixture was adjusted to its initial pH of 7.0 and then added to the homogenized diet.

clams resulted in rapid and complete destruction of the added vitamin. Incubation of this dietary mixture likewise failed to lower the residual thiamine content. It may very well be that of the total thiamine figure for the basal dietary mixture 0.2 mg., or 15%, represents non-specific reacting substances indistinguishable from thiamine in the thiochrome assay procedure. These experiments demonstrate that within 20 minutes one serving of clams (100 gm.) can destroy more than 8 mg. of thiamine, i.e., more than eight times the minimal daily requirment. However, factors capable of destroying ingested enzymes or of rendering them inactive are known to be present in the gastrointestinal tract. The effect of the ingestion of raw clams on the availability of thiamine, when disintegration of cells and liberation of the thiaminase occurs by virtue of

the digestive enzymes in the gastrointestinal tract, could only be determined by direct studies on man.

In vivo studies. The results of the study on human test subjects are presented in table 3. The control data were obtained with the individuals on the basal ration containing the heat-inactivated clam suspension.

During the first 3 days no extra thiamine was taken. On the fourth day a test dose of 7.5 mg. of the vitamin was administered postprandially, and on the fifth day the basal ration alone was again ingested. The responses of the subjects were quite uniform; good reproducibility was

TABLE 3

Urinary excretion of dietary and extra thiamine by subjects subsisting on a diet containing raw and heated[1] clams.

(All values in milligrams.)

SUBJECT	DIET CONTAINING HEATED CLAMS					NORMAL BASAL EXCRETIONS[2]	DIET CONTAINING RAW CLAMS[3]				
	Basal excretions			After 7.5 mg. of thiamine			Basal excretions			After 7.5 mg. of thiamine	
	1st 24 hrs.	2nd 24 hrs.	3rd 24 hrs.	1st 24 hrs.	2nd 24 hrs.		1st 24 hrs.	2nd 24 hrs.	3rd 24 hrs.	1st 24 hrs.	2nd 24 hrs.
J.C.	0.22	0.23	0.23	1.59	0.54	0.23	0.12	0.11	0.10	0.88	0.16
E.M.	0.25	0.19	0.19	1.33	0.33	0.21	0.12	0.09	0.07	0.59	0.25
D.M.	0.25	0.29	0.28	1.80	0.47	0.27	0.17	0.11	0.11	0.74	0.25
M.H.	0.19	0.24	0.22	1.02	0.38	0.22	0.15	0.14	0.11	0.85	0.25
H.H.	0.32	0.28	0.26	1.79	0.36	0.29	0.14	0.09	0.09	0.95	0.18
Average	0.25	0.25	0.24	1.51	0.42	0.24	0.14	0.11	0.10	0.80	0.22

[1] An aqueous suspension of ground clams at pH 4.5 boiled to inactivate the thiaminase.

[2] The average excretion of the corresponding subject subsisting on the diet containing the heated clams.

[3] The raw clams were served whole.

observed from day to day in their basal thiamine excretion, as well as an increased output on the day following the test dose and small carry-over (second 24 hour) values. Two weeks later the experiment was repeated, but this time the diet included the clams in their natural raw state.[5] In every case there was a prompt and continuous decrease in the basal urinary excretion values as the subjects continued on the diet containing the raw clams. The increments in the urinary excretion values following dosage with 7.5 mg. of thiamine were consistently smaller than those noted when the thiamine solution was taken along with the heated clams. This relationship was also apparent in the carry-over values for the two periods.

[5] The clams were chewed two or three times, as is customarily done, leaving disintegration of the cells and liberation of the thiaminase to gastrointestinal enzymic processes.

Table 4 is an evaluation of the data of the preceding table in order to obtain a quantitative index of the destructive action of the thiaminase in raw clams during gastrointestinal digestion. The decrease in the basal thiamine excretion values would indicate that 50% or more of the dietary thiamine is destroyed. This urinary excretion picture is comparable to that obtained with normal subjects during the first 3 days following a 70% reduction in the normal thiamine intake (Melnick, Field and Robinson, '39).[6] When the extra urinary responses for the control and test period are compared on the basis of per cent of test dose excreted, 42% destruction of the added thiamine is found to have occurred.

TABLE 4

Destruction of dietary and extra thiamine by thiaminase in clams.

SUBJECT	DIET CONTAINING HEATED CLAMS			DIET CONTAINING RAW CLAMS				
	Average basal excretion	Fraction of 7.5 mg. test dose excreted		Change in basal excretion [1]			Fraction of 7.5 mg. test dose excreted	
		1st 24 hrs.	2nd 24 hrs.	1st 24 hrs.	2nd 24 hrs.	3rd 24 hrs.	1st 24 hrs.	2nd 24 hrs.
	mg./24 hrs.	%	%	%	%	%	%	%
J.C.	0.23	18.1	4.1	— 46	— 50	— 56	10.3	0.8
E.M.	0.21	14.8	1.5	— 44	— 56	— 66	6.9	2.3
D.M.	0.27	20.4	2.6	— 36	— 58	— 60	8.4	1.9
M.H.	0.22	10.8	2.2	— 30	— 37	— 48	9.8	1.8
H.H.	0.29	20.0	0.9	— 53	— 68	— 68	11.4	1.2
Average	0.24	16.8	2.3	— 42	— 55	— 60	9.4	1.6
Average total excretion of extra thiamine		19.1%		..	..	..	11.0%	
Destruction of thiamine taken as test dose	$\frac{19.1-11.0}{19.1} \times 100 = 42\%$.							

[1] Based on the average excretions of the corresponding subjects receiving the diet containing the heated clams.

DISCUSSION

The extent of thiamine destruction in vivo is not as great as that observed in the in vitro experiments. This is undoubtedly due to the fact that in the latter tests the clams were added to the dietary mixture in a finely homogenized state, allowing immediate and more complete contact between thiamine and thiaminase in the static system. In the study with the human subjects the thiaminase was liberated slowly as the clams were digested by enzymic processes. Thus, the opportunity existed for absorption of some dietary thiamine and certainly of a significant fraction of the test dose prior to the liberation of appreciable amounts of the thiaminase.

[6] Subject, D. M., participated in both studies.

The effectiveness of the thiaminase in clams in destroying thiamine and the presence of this factor in other fish products (such as the herring) ordinarily consumed in the raw state represent a type of conditioning factor potentially responsible for malnutrition (Jolliffe, '43). The present study is far from being a survey of all fish products intended for direct human consumption, although it might be worthy of extension especially in relation to populations in those regions where fish, raw or partially cooked, constitute a major element of the diet. In any case, these observations indicate that the presence of the anti-thiamine factor in raw fish is not merely of academic interest but worthy of practical consideration in human nutrition.

The anti-thiamine effect of the homogenized clam suspension should find applications in methods, biological and chemical, designed to measure thiamine. In the former type of assay, the basal diet must supply all factors except thiamine. Autoclaving or sulfite treatment (U. S. Pharmacopoeia, '42) of the ingredients which furnish the B complex to insure complete destruction of thiamine, often involves concomitant destruction of other factors. The materials may preferably be incubated with a homogenized clam suspension to effect specific destruction of the thiamine. The blank value in the thiochrome procedure for determining thiamine (Hennessy, '41; U. S. Pharmacopoeia, '44) is frequently unsatisfactory since fluorescent materials may be present in the alkalinized solution which are destroyed on the addition of ferricyanide (Najjar and Ketron, '44). The sulfite treatment of the test solution (Mason and Williams, '42) has been reported to destroy interfering substances as well as thiamine thereby yielding low blanks (Najjar and Ketron, '44). However, the short incubation of a test extract with a small amount of an aqueous homogenized clam suspension (which is always free from thiamine) should allow specific destruction of thiamine leaving the true blank correction.

SUMMARY

In vitro tests have indicated that various fish products intended for direct human consumption contain a thiaminase which destroys thiamine in a static test system. Rapid and complete destruction of the vitamin occurred in vitro during incubation of food mixtures containing added thiaminase, as present in raw clams. More than eight times the minimal daily requirement of thiamine was destroyed simply in preparing a homogeneous mash of a daily ration to which both clams and thiamine had been added.

The results of the human availability study indicate that an appreciable destruction of thiamine (about 50%) occurs in the gastrointestinal tract following the concomitant ingestion of raw clams. Because other fish products are also consumed in a raw or partially cooked state by man, this finding warrants consideration of the anti-thiamine principle in fish products as a possible conditioning factor in malnutrition.

The application of the thiaminase in clams to vitamin B_1 methodology, both biological and chemical, has been discussed.

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COMPARATIVE MERITS OF FASTING SPECIMENS, RANDOM SPECIMENS AND ORAL LOADING TESTS IN FIELD NUTRITIONAL SURVEYS [1]

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ONE FIGURE

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The systematic carrying out of nutritional observations on soldiers in the field is frequently difficult for military reasons, and it is at times almost impossible to collect samples of blood and urine under conditions and at times that are customarily considered almost essential for accurate nutritional assessment. Of the usual types of specimen,[2] one can almost always obtain fasting urine and blood before breakfast and at other times of day; sometimes conduct short vitamin loading tests; and almost never collect specimens of urine for periods of 24 hours. Many practical problems of conducting surveys would be made simpler if one knew accurately the quantitative relations between specimens collected before breakfast, after loading tests, and at random during the day.

The present study was carried out on soldiers living and working in temperature environments while subsisting on field rations, and the data support four main conclusions concerning men under such conditions. First, loading tests with ascorbic acid, thiamine, riboflavin and

[1] This work was financed in part under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the President and Fellows of Harvard College. The data were obtained in U. S. Army field trials to which the senior author was assigned as expert consultant for the Research and Development Branch, Military Planning Division, Office of the Quartermaster General. Permission to publish these findings has been granted by the Bureau of Public Relations, U. S. War Department, but the assertions and opinions expressed are those of the authors, and do not necessarily reflect the official position of any governmental agency.

[2] Throughout this paper several conventions of nomenclature will be followed. "Fasting" and "postabsorptive" are used synonymously to mean samples collected between the time of emptying the bladder on arising but before breakfast. "Postprandial" and "postcibal" are used synonymously to mean samples collected at any time after breakfast but before bedtime. "Loaded" refers to specimens collected after the administration of test doses of vitamins.

nicotinamide could be repeated on the same subjects at intervals of 2 or 3 weeks without significantly affecting their response to subsequent loading tests. Second, the rate of excretion of vitamins in the morning before breakfast was a more reliable indication of previous intake of vitamins than was the vitamin load test. Third, specimens of blood drawn at any time of day were directly comparable in several important respects with specimens drawn before breakfast. Fourth, even when due allowance was made for rate of urinary flow or urinary specific gravity samples of urine collected at various times of day did not give results for chloride, ascorbic acid, thiamine, N^1-methylnicotinamide or riboflavin that were directly comparable with results from samples collected before breakfast.

METHODS

Samples of blood and urine were collected in bivouac areas and analyses were carried out on the spot in a field laboratory. Whole blood hemoglobin was estimated by the copper sulfate specific gravity method of Phillips, Van Slyke and colleagues ('43); serum protein by the same method; serum and urinary chloride according to Harvey ('10); serum and urinary ascorbic acid according to Farmer and Abt ('36); and urinary thiamine, N^1-methylnicotinamide and riboflavin by fluorometric methods (Johnson, Sargent, Robinson and Consolazio, '45).

Observations were made on two separate groups under different conditions of diet, environment and activity which will be differentiated below. The subjects were all in good general health as judged by their medical officers.

RESULTS

A. Effects of repeated load tests

These were studied in a company (96 men) from an infantry battalion that lived and worked for 8 weeks in the summer in a bivouac area situated in the Rocky Mountains at an altitude of about 9000 feet. The daily temperatures ranged from near freezing at dawn to about 90°F. in the heat of the day, with infrequent thunder showers interrupting the otherwise clear, sunny weather. Activities included regular infantry training, cross-country marches, road marches, and tactical problems, with 1 day of rest a week, the whole summer's program involving an estimated average daily work expenditure of about 4000 Cal.

The soldiers all subsisted exclusively on a combat ration providing the following estimated average daily amounts of vitamins: ascorbic

acid, 65 mg.; riboflavin, 1.5 mg.; thiamine, 2.0 mg.; and niacin, 17 mg. Vitamin load tests were administered to 50 of the subjects on the first day of maneuvers, again 3 weeks later and finally at the end of 5 weeks. A vitamin load test was administered to the remaining forty-six subjects only once, at the end of the 5 weeks. The two groups were large enough so that comparison between them could be made on the assumption that their average nutritional status was the same except for the difference in load tests.

On test days fasting specimens of urine were collected between 4:45 A.M. and 6:15 A.M. and each subject then drank 150 ml. of water containing 5 mg. thiamine hydrochloride, 5 mg. riboflavin, 50 mg. nicotinamide, and 500 mg. ascorbic acid. Their usual breakfast was allowed and the subjects collected all their urine for 4 hours after the test dose. We use this schedule for tolerance tests in the field because breakfast has to be allowed for the sake of morale and a good morning's

TABLE 1

Fasting urinary levels in men who had and had not been subjected previously to vitamin load tests.

	NO PREVIOUS LOAD TEST (46 SUBJECTS)		LOAD TESTS 5 AND 2 WEEKS PREVIOUSLY (50 SUBJECTS)	
	Mean	*Range*	*Mean*	*Range*
Ascorbic acid (mg./hr.)	0.6	0.4 to 1.8	0.8	0.4 to 1.8
Thiamine (μg./hr.)	11	6 to 24	12	6 to 42
Riboflavin (μg./hr.)	27	12 to 68	22	8 to 93
N^1-methylnicotinamide (mg./hr.)	0.25	0.10 to 0.50	0.30	0.10 to 0.58

performance; furthermore intravenous tests on a large scale are not feasible in the field; also longer collection periods are usually impossible because of military necessities. The reliability of the 4-hour oral test in the case of vitamin C has been completely demonstrated for groups of subjects whose body stores were known to be very low, average and high by virtue of 8 weeks' subsistence on diets lacking, normal or high in vitamin C (Johnson, Darling, Sargent and Robinson, '45); and its validity in the case of thiamine, riboflavin and nicotinamide is demonstrated in a forthcoming paper (Johnson, Contreras, Robinson and Consolazio, '45).

The results for fasting urine, converted to hourly excretions from those of the actual collection periods, are summarized in table 1. The averages for ascorbic acid, thiamine and N^1-methylnicotinamide were not significantly different between the two groups and the average for riboflavin was higher in the group that had never previously received a load test. The range tended to extend a little higher in the group

that had received two previous load tests, but the distribution curves were not significantly different, except that riboflavin values were somewhat higher in the group that had never received load tests.

The comparison of the load tests was similar to that of the fasting specimens collected before breakfast. The averages for the groups that had and had not received load tests were: for ascorbic acid — 54 and 64 mg. in 4 hours after the test dose; for thiamine — 405 and 400 μg. in 4 hours; for N^1-methylnicotinamide — 4.75 and 4.75 mg. in 4 hours; and for riboflavin — 1575 and 1600 μg. in 4 hours. Inspection of the distribution curves (fig. 1) shows a fairly wide spread in both groups, but curves that are very similar in shape.

From this set of observations, it may be concluded that in the case of men on similar diets of reasonably high vitamin content, assays of

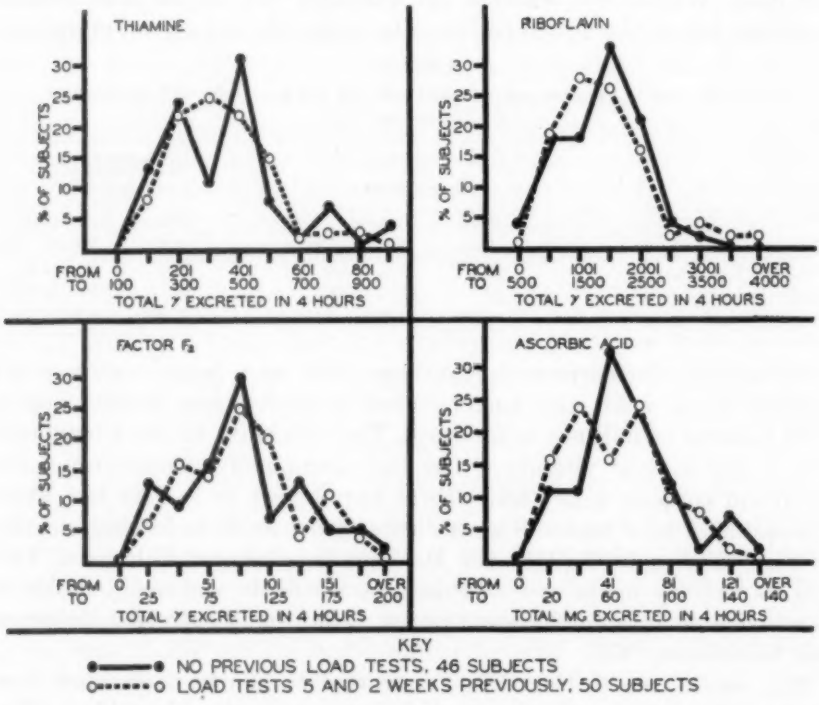
THIAMINE
% OF SUBJECTS
FROM TO 0 100 201 300 401 500 601 700 801 900
TOTAL γ EXCRETED IN 4 HOURS
RIBOFLAVIN
% OF SUBJECTS
FROM TO 0 500 1001 1500 2001 2500 3001 3500 OVER 4000
TOTAL γ EXCRETED IN 4 HOURS
FACTOR F2
% OF SUBJECTS
FROM TO 0 1 25 51 75 101 125 151 175 OVER 200
TOTAL γ EXCRETED IN 4 HOURS
ASCORBIC ACID
% OF SUBJECTS
FROM TO 0 1 20 41 60 81 100 121 140 OVER 140
TOTAL MG EXCRETED IN 4 HOURS
KEY
NO PREVIOUS LOAD TESTS, 46 SUBJECTS
LOAD TESTS 5 AND 2 WEEKS PREVIOUSLY, 50 SUBJECTS

Fig. 1 Distribution curves for urinary excretion of thiamine, riboflavin, N^1-methylnicotinamide and ascorbic acid after oral test doses in subjects who had and had not previously received such tests. Ordinates are the percentage of subjects in categories represented by abscissae, which are the ranges of urinary excretion in 4 hours after test dose. (The factor for converting values for Factor F_2, which are expressed in micrograms, quinine units, into N^1-methylnicotinamide, milligrams, is $1/20$.)

the fasting urine before breakfast and of oral load tests of short duration are not significantly affected by load tests administered at intervals of 2 or 3 weeks. This conclusion should not be extended without further evidence to subjects on frankly deficient diets or to types of load test different from that used in this study.

B. Comparison of fasting urinary levels with results of load tests

This comparison was made between two platoons (26 and 31 men, respectively) of a company from the battalion described in Section A above. The diet was a field ration which provided the following estimated daily average intake of nutrients: total calories, 4100; protein, 140 gm.; ascorbic acid, 90 mg.; riboflavin, 2.5 mg.; thiamine, 2.1 mg.; and niacin, 27 mg. During the last 3 weeks of the test, platoon 1 received placeboes and platoon 2 vitamin pills providing daily: vitamin A, 7500 U.S.P. units; vitamin D, 600 U.S.P. units; ascorbic acid, 112.5 mg.; thiamine hydrochloride, 3.0 mg.; riboflavin, 4.5 mg.; and nicotinamide, 30.0 mg.

Under the present experimental circumstances the fasting specimen was a far more sensitive indication that the subjects had been receiving supplementary ascorbic acid, riboflavin, thiamine, and nicotinamide than was the oral load test (table 2). Highly significant differences be-

TABLE 2

Comparison between platoon 1, which received placeboes for 3 weeks, and platoon 2, which received vitamin pills.

	MEANS FOR PLATOON 1 (26 MEN)		MEANS FOR PLATOON 2 (31 MEN)	
	Before	Placeboes for 3 weeks	Before	Vitamin pills for 3 weeks
A. Urinary excretion in fasting state [1]				
Ascorbic acid (mg./hr.)	0.8	0.8	0.8	1.4
Thiamine (μg./hr.)	17	12	14	23
N^1-methylnicotinamide (mg./hr.)	0.30	0.30	0.21	0.38
Riboflavin (μg./hr.)	21	26	23	57
B. Excretion after load tests [2]				
Ascorbic acid (mg./4 hr.)	61	103	59	113
Thiamine (μg./4 hr.)	670	530	600	575
N^1-methylnicotinamide (mg./4 hr.)	7.25	6.55	5.25	4.20
Riboflavin (μg./4 hr.)	975	1860	1115	2195

[1] Analysis of variance shows $P < 0.01$ that the difference between the platoons during the 3 weeks is chance for all measurements in A.

[2] Analysis of variance shows $P < 0.06$ or more that the difference between the platoons during the 3 weeks is chance for all measurements in B.

tween platoons were demonstrated in the fasting rates of excretion of ascorbic acid, thiamine, riboflavin and N^1-methylnicotinamide. The results for the load tests showed far less statistical significance than those for the fasting specimens. There did tend to be mean differences between the platoons in the load tests for ascorbic acid, thiamine and riboflavin, but for N^1-methylnicotinamide the unsupplemented platoon showed less of a decrease than did the supplemented. This latter finding is not surprising in view of the known qualifications that must be placed on the interpretation of N^1-methylnicotinamide levels, owing to extreme individual variations in response to test doses of nicotinamide (Sargent, Robinson and Johnson, '44).

The fasting rate of excretion of thiamine, riboflavin and N^1methylnicotinamide has been widely used in the assessment of nutritional deficiencies (Najjar and Holt, '42), and the present observations suggest that even in well-fed young men these observations may be at least as reliable as some kinds of load test. We shall turn now to a comparison of fasting levels with postcibal.

C. Levels in blood and urine at different times of day

For these comparisons the subjects were twelve men isolated for 2 weeks on a barren, windswept island in Buzzard's Bay, Massachusetts. The weather was fine but windy and cool, with an overall range of temperature from $+43°$F. to $+57°$F. Activities included maintaining camp, conducting laboratory work, and engaging in moderate activity outdoors, with an estimated average daily work expenditure of about 3500 Cal.

They subsisted throughout on a field ration that provided a variety of components but essentially constant daily intake of nutrients as shown in table 3.

Samples of venous blood were drawn every morning immediately after reveille but before breakfast, and timed specimens of urine were obtained at the same period. Postprandial specimens were collected at other times of day, so that by the end of 12 days comparisons had been made between fasting levels and levels in midmorning, early afternoon, late afternoon and after supper.

1. Results for blood (table 4). Serum protein and chloride showed but slight changes, with a tendency to a diurnal increase. Hemoglobin remained almost unchanged except for a small unexplained decrease during the ninth day. Serum ascorbic acid showed increases which were small when expressed in absolute amounts, but were large on a percentage basis. In view of the relatively large intake of ascorbic

acid at the noon meal (table 3) the smallness of the diurnal change was somewhat surprising. The general conclusion for blood is that under the present circumstances assessment of hemoglobin, serum protein, serum chloride and serum ascorbic acid could be made satisfactorily from samples taken at any time of day.

TABLE 3

Average daily consumption of second group of test subjects.

	BREAKFAST 7 A.M.	DINNER 12 NOON	SUPPER 5:30 P.M.	TOTAL FOR WHOLE DAY
Total Calories	1700	1270	1680	4650
Protein (gm.)	54	11	64	129
Ascorbic acid (mg.)	0	70	6	76
Thiamine (mg.)	0.9	0.4	0.6	1.9
Riboflavin (mg.)	1.2	0.1	1.4	2.7
Niacin (mg.)	8.6	2.7	11.4	22.7

TABLE 4

Fasting levels in whole blood and serum compared with levels at other times of day (average of 12 subjects).

TEST DAY	SPECIMEN	HEMOGLOBIN	SERUM PROTEIN	SERUM CHLORIDE	SERUM ASCORBIC ACID
		gm./100 m'.	*gm./100 m'.*	*meq./l.*	*mg./100 ml.*
3	9 A.M.	15.5	6.5	105	..
5	Fasting, 7 A.M.	15.4	6.4	103	0.7
5	1 P.M.	15.3	6.5	104	0.9
5	Δ	− 0.1	+ 0.1	+ 1	+ 0.2
7	Fasting, 7 A.M.	15.4	6.8	102	0.5
7	3 P.M.	15.6	6.8	103	0.6
7	Δ	+ 0.2	0	+ 1	+ 0.1
9	Fasting, 7 A.M.	15.8	6.4	101	0.4
9	7:30 P.M.	15.2	6.8	102	0.6
9	Δ	− 0.6	+ 0.4	+ 1	+ 0.2

2. Results for urine (table 5). The urinary data should be examined with a view to obtaining from random specimens those values which are directly related to fasting levels. Preferably one would desire a reasonable approximation of the order of magnitude of ± 10% of the fasting value, but any constant relationship would satisfy the present requirements. However, neither expressing the results as concentration per 100 ml. of urine (table 5B), nor as concentration per 100 ml. of urine, corrected to specific gravity 1.010 (table 5C), nor as excretion per hour (table 5D) allowed chloride, ascorbic acid, thiamine, N^1-methylnicotinamide or riboflavin in random specimens to be calculated

TABLE 5

Fasting urinary levels compared with levels at other times of day. (Average of 12 subjects on constant diet.)

MEASUREMENT	DAY AND SPECIMEN											
	Day 3			Day 5			Day 7			Day 9		
	F[1]	11 A.M.	Δ. %F[1]	F[1]	3 P.M.	Δ. %F[1]	F[1]	9 P.M.	Δ. %F[1]	F[1]	6 P.M.	Δ. %F[1]
A. Rate of urinary flow and specific gravity of urine												
Urine volume (ml./hr.)	44	91	+ 107	36	92	+ 155	46	57	+ 24	44	99	+ 125
Urine specific gravity	1.026	1.021	— 0.5	1.026	1.017	— 0.9	1.026	1.023	— 0.3	1.023	1.024	+ 0.1
B. Excretion per 100 ml. urine												
Chloride (gm. NaCl/100 ml.)	1.20	1.17	— 3	1.20	0.70	— 42	1.06	1.02	— 4	1.23	1.05	— 15
Ascorbic acid (mg./100 ml.)	1.4	1.7	+ 21	1.7	1.6	— 6	1.1	1.6	+ 46	1.1	1.5	+ 36
Thiamine (μg./100 ml.)	9	9	0	8	9	+ 13	9	12	+ 33	11	8	— 27
N^1-methylnicotinamide (mg./100 ml.)	0.80	0.75	— 6	1.10	0.60	— 45	0.45	0.60	+ 33	0.80	0.60	— 25
Riboflavin (μg./100 ml.)	82	33	— 60	86	27	— 69	62	56	— 10	55	30	— 45
C. Excretion per 100 ml. urine, calc. to Sp. G. 1.010												
Chloride (gm. NaCl/100 ml.; Sp.G. 1.010)	0.46	0.55	+ 20	0.46	0.41	— 11	0.41	0.44	+ 7	0.54	0.44	— 19
Ascorbic acid (mg./100 ml.; Sp.G. 1.010)	0.5	0.8	+ 60	0.7	0.9	+ 29	0.4	0.7	+ 75	0.5	0.6	+ 20
Thiamine (μg./100 ml.; Sp.G. 1.010)	3	4	+ 33	3	5	+ 67	3	5	+ 67	5	3	— 40
N^1-methylnicotinamide (mg./100 ml.; Sp.G. 1.010)	0.30	0.35	+ 17	0.40	0.35	— 13	0.15	0.25	+ 67	0.35	0.25	— 29
Riboflavin (μg./100 ml.; Sp.G. 1.010)	32	16	— 50	33	15	— 55	24	25	+ 4	24	13	— 46
D. Excretion per hour												
Chloride (gm. NaCl/hr.)	0.53	1.06	+ 100	0.37	0 64	+ 73	0.49	0.58	+ 18	0.54	1.04	+ 93
Ascorbic acid (mg./hr.)	0.6	1.5	+ 150	0.5	1.5	+ 200	0.5	0.9	+ 80	0.5	1.5	+ 200
Thiamine (μg./hr.)	4	8	+ 100	3	8	+ 167	4	7	+ 75	5	8	+ 60
N^1-methylnicotinamide (mg./hr.)	0.35	0.70	+ 100	0.35	0.55	+ 57	0.20	0.35	+ 75	0.35	0.60	+ 71
Riboflavin (μg./hr.)	36	30	— 17	27	25	— 7	29	32	+ 10	24	30	+ 25

[1] Represents specimens collected between the time of emptying bladder on arising and the time of breakfast.

so as to be even roughly comparable with fasting values. The nearest approaches were for chloride when expressed as concentration per 100 ml. urine (2 out of 4 values) and for riboflavin when expressed as excretion per hour (2 out of 4 values). The latter finding is at variance with the interpretation put on their data by Feder, Lewis and Alden ('44) who found much more constancy when urinary riboflavin was expressed in terms of actual concentration than when it was calculated on an hourly basis. Their experimental conditions and ours were so widely different that this discrepancy is not disturbing.

DISCUSSION

The present results are applicable to active young men subsisting on a relatively constant diet, such as field rations, and are not necessarily valid for other types of population or diets, particularly deficient ones. With these reservations in mind, we can draw three conclusions of practical importance in conducting nutrition surveys when chloride, ascorbic acid, thiamine, N^1-methylnicotinamide, riboflavin, hemoglobin and serum protein are in question. First, if it is not possible to conduct all the tests desired, preference should be given to obtaining fasting specimens rather than to conducting load tests. Second, under the same conditions and in temperate environments, specimens of blood give reasonably comparable results regardless of the time of day. Finally, it is highly desirable to collect fasting rather than random specimens of urine, owing to the difficulty in obtaining values otherwise even roughly comparable with fasting levels.

SUMMARY

1. Repetition of 4-hour oral vitamin loading tests employing ascorbic acid, thiamine, riboflavin and nicotinamide administered at intervals of 2 or 3 weeks to soldiers subsisting on good field rations had no significant effects on the results of subsequent loading tests or on the fasting urinary excretion of ascorbic acid, thiamine, riboflavin or N^1-methylnicotinamide.

2. Under the same circumstances the fasting rates of urinary excretion of ascorbic acid, thiamine, riboflavin and N^1-methylnicotinamide were more sensitive measures of vitamin intake in the previous 3 weeks than were 4-hour oral vitamin loading tests.

3. Whole blood hemoglobin serum protein, serum chloride and serum ascorbic acid varied relatively little in the course of the day.

4. In contrast to the results for blood and serum, the fasting urinary rates of excretion of chloride, ascorbic acid, thiamine, N^1-methylnicotin-

[1] Represents specimens collected between the time of emptying bladder on arising and the time of breakfast.

amide and riboflavin and values obtained at other times of day were not satisfactorily related in a simple manner either when expressed: (a) as concentration per 100 ml. of urine, (b) as concentration per 100 ml. of urine corrected to specific gravity 1.010, or (c) as excretion per hour.

5. The practical conclusions for nutritional surveys are that when it is not possible to conduct all the tests that might be desired, fasting levels should be determined in preference to the results of loading tests; that specimens of blood may with reservations be drawn at any time of day; and that random specimens of postprandial urine should be avoided if possible. These conclusions are limited to hemoglobin, serum protein, chloride and ascorbic acid; and to urinary chloride, ascorbic acid, thiamine, N^1-methylnicotinamide and riboflavin.

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EFFECTS OF FOOD INTAKE AND ANOXIA UPON ASCORBIC ACID EXCRETION, ACIDITY OF URINE, AND SURVIVAL OF MALE ALBINO RATS[1]

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The present investigation was undertaken because of the need for experimental data concerning (a) the effect of food intake upon optimal resistance to anoxia and (b) the trend of reactions that are induced by lowered atmospheric pressure. Concurrent studies with human subjects gave additional evidence of the importance of food composition in relation to altitude tolerance.

Evidence has been presented by Packard ('07), Boothby et al. ('40), Polonovski ('40), McFarland ('41) and others, that a diet high in carbohydrates can afford measurable protection against anoxia. That carbohydrate metabolism in turn, is affected by exposure to anoxia has been demonstrated also (Van Middlesworth et al., '44; Bryan et al., '44; Warren, '41; McQuarrie et al., '42). Campbell ('38) concluded that the diet which gives greatest protection against acute anoxia in white rats is one made up solely of carrots. Many of his observations were confirmed by Nelson et al. ('43). Diets found by Campbell to give no protection against anoxia included raw and cooked horse meat, cheese, and whole milk, all of which are excellent sources of protein. Contrary to these findings, Bierman ('43) suggested, although without supporting experimental data, that a diet high in protein is preferable. There are sound theoretical reasons, however, for believing that preflight and inflight meals high in protein would impair altitude tolerance, and carefully controlled observations with human subjects have demonstrated there is such a relationship (King et al., '45; Butts, Mulholland and Green, '45).

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[2] The experimental data given in the present manuscript were presented by one of the authors (K. E. L.) in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutrition, under the Joint Committee on Graduate Instruction, Columbia University.

EXPERIMENTAL

In this investigation three specific alterations in diet were studied. One group of rats was given a diet of Purina dog chow ad libitum and a daily supplement of 5 gm. of raw horse meat. A control group was continued on the dog chow diet alone. Second, two groups of rats were fed dog chow ad libitum, but a daily supplement of 2 gm. of sucrose in syrup form was fed to one group. Third, one group of rats was fed dehydrated carrots ad libitum as the sole source of food and the control group was fed an isocaloric quantity of dog chow. Before determining the relative survival rates when subjected to severe anoxia (3% of oxygen, 97% of nitrogen), composite urine samples were collected from each group during three periods for the determination of pH, titratable acidity, and ascorbic acid. During the first period, all animals were on the chow diet; during the second, one group was on the chow diet and the other received chow plus the supplement; during the third period, experimental diets were continued and the animals were subjected to mild anoxia (7.7% of oxygen, 92.3% of nitrogen).

Apparatus. To permit collection of composite urine samples, the animals were placed in long narrow cages divided into eight compartments, each of which accommodated two rats. The cages were equipped with stainless steel wire screen bottoms and were placed over glass-lined funnels. Mild anoxia (simulated altitude, 25,000 feet) was produced by exposing the animals to a gas mixture containing 7.7% of oxygen and 92.3% of nitrogen at atmospheric pressure.

Analytical procedure. On the days that ascorbic acid content of the urine was to be determined, the urine was collected in metaphosphoric acid of such strength that the final concentration was approximately 3%. The ascorbic acid content was then determined by use of a Coleman Universal Spectrophotometer, Model 11, following the procedure recommended by Bessey ('38) for the determination of small amounts of ascorbic acid in turbid solutions in the presence of other reducing substances.

Titratable acidity was determined on the composite urine samples according to Folin's method. A Beckman pH meter, Laboratory Model G, was used to determine pH values.

Experimental animals. Male albino rats from Professor Sherman's well standardized colony were used. Any animals that did not show a normal gain in weight after being weaned (28 days) and placed on a regimen of Purina dog chow (proximate analysis: carbohydrate 48.5, protein 26.2, fat 5.4%) and tap water ad libitum were discarded.

Protein tests. (A) Fifteen pairs (litter-mates) of male albino rats were divided into two groups in such a way that the average age of the rats in each group was 88 days and the average body weight was 210 gm. For a period approximately 2 weeks, both groups were continued on the chow diet. Composite urine samples were collected over a 3-hour period, during which time the animals did not have access to either food or water. Samples for pH and titratable acidity measurements and for ascorbic acid determinations were collected on successive days.

TABLE 1

Results (averages) obtained during three successive periods of two protein feeding tests: (1) all animals on chow diet; (2) group I on chow diet and group II on meat supplement; (3) all animals subjected to mild anoxia.

	ASCORBIC ACID				ACID-BASE DATA					
	Volume				Volume		pH		N/10 NaOH per ml.	
	Groups				Groups		Groups		Groups	
	I	II	I	II	I	II	I	II	I	II
	ml.		*µg./ml.*		*ml.*		*Range*		*ml.*	
				A. First series:						
1st period (3 hrs.)	13.2	12.8	37	45	11.1	11.6	6.3–7.0	6.6–7.7	0.44	0.30
2nd period (3 hrs.)	15.1	25.8	56	51	13.9	24.0	6.1–6.7	6.6–7.1	0.56	0.39
3rd period (2 hrs.)	44.1	52.5	10	6	48.1	55.6	7.2–7.6	7.4–7.8	0.10	0.06
				B. Second series:						
1st period (6 hrs.)	12.4	11.1	38	37	12.1	11.3	5.3–7.5	5.6–7.9	0.36	0.34
2nd period (6 hrs.)	13.2	20.6	31	21	14.7	20.5	5.4–7.0	6.1–7.5	0.57	0.34
3rd period (3 hrs.)	35.5	52.4	4	2	32.4	49.5	8.1–8.3	8.6–8.6	0.00	0.00

During a 16-day period, the diet of group I was unaltered, and group II was given a supplement of 5 gm. of raw horse meat half an hour before the collection of the composite urine sample. Both groups were subjected to mild anoxia during the following week. After obtaining the ascorbic acid, pH and titratable acidity values on 3 different days, the animals were subjected to severe anoxia (5% oxygen, 95% nitrogen). Six of the chow-fed group and two of the meat-fed group survived this treatment. In table 1 will be found the average results of analysis of six composite urine samples collected during the preliminary and supplement-feeding periods and of three samples collected during the period of mild anoxia. The average body weights of the control and meat-fed groups were, respectively: 210 and 210 gm. at the beginning, 232 and 231 gm. when the supplement was started; and 254 and 264 gm. at the end.

(B) Because of the wide variations in results obtained by analysis of composite urine samples collected while the animals were held under comparable conditions, the experiment outlined in (A) was repeated, with slight changes. Urine samples were collected from sixteen individual male rats (average age, 86 days; average weight, 154 gm.) over a 6-hour interval. The samples were titrated with 2,6-dichlorophenol indophenol to an endpoint which lasted approximately 5 seconds. The sixteen rats were then divided into two groups in such a way that the average ascorbic acid excretion per milliliter of urine was approximately the same for each group. During the next 2-week period, a supplement of 5 gm. of raw horse meat was fed daily (with the exception of Saturdays and Sundays) to each animal in group II. Urine samples from the individual rats were collected during the period immediately after consumption of the meat. Titratable acidity and pH, and ascorbic acid determinations were made on alternate days. Composite urine samples were collected during a 3-hour exposure to mild anoxia. Average values are given in the B section of table 1 for the individual samples collected during 4 days in the first and second periods of the experiment and on the composite samples collected during the period of mild anoxia. The average initial, intermediate and final body weights of the animals in groups I and II were, respectively: 156 and 151 gm.; 169 and 172 gm.; and 204 and 229 gm. The two groups of rats were then subjected to severe anoxia for an hour and three-quarters. Of the meat-fed group, one survived; of the chow-fed group, four survived.

Carbohydrate tests. (A) Thirty male albino rats, average age 111 days and body weight 248 gm., were divided into two groups of fifteen each. During the preliminary period, 3-hour composite urine samples were collected from each group and analyzed. The animals in one group were then given, by means of a medicine dropper, 2 gm. of sucrose in syrup form (66.6% by weight) each day immediately before collecting the 3-hour urine sample (second period). Both groups of rats were then subjected to mild anoxia for 2 hours (third period). One rat in the chow-fed group and four in the sugar-fed group did not survive this treatment. The 2-hour exposures to mild anoxia and supplement feeding were continued, and the data obtained on ascorbic acid and titratable acidity were calculated to a basis of fifteen rats in each group. When the two groups were subjected to severe anoxia, five rats in each group survived. The average values obtained on analysis (a) of six samples of urine collected during the preliminary and supplement-feeding periods and (b) of five samples collected during the period

of anoxia are given in table 2. The intermediate and final average body weights of groups I and II were 251 and 249, and 273 and 265 gm., respectively.

TABLE 2

Results (averages) obtained during three successive periods of three series of carbohydrate feeding tests: (1) all animals on chow diet; (2) group I on chow diet and group II on sugar supplement; (3) all animals subjected to mild anoxia.

	ASCORBIC ACID				ACID-BASE DATA				N/10 NaOH per ml.	
	Volume		Concn.		Volume		pH			
	Groups		Groups		Groups		Groups		Groups	
	I	II	I	II	I	II	I	II	I	II
	ml.		μg./ml.		ml.		Range		ml.	
A. First series:										
1st period (3 hrs.)	14.1	14.6	31	48	14.5	15.6	6.1–7.0	6.3–7.4	0.52	0.46
2nd period (3 hrs.)	14.8	12.3	34	77	15.1	12.2	6.3–6.7	6.0–6.2	0.49	0.60
3rd period (2 hrs.)	62.7	64.5	7	15	53.2	51.1	6.9–7.5	7.0–7.6	0.09	0.09
B. Second series:										
1st period (4 hrs.)	16.7	16.3	87	86	15.7	15.3	6.0–6.3	6.2–6.5	0.69	0.62
2nd period (4 hrs.)	15.1	14.5	80	86	16.4	16.3	6.1–6.4	6.2–6.6	0.55	0.45
3rd period (2 hrs.)	39.4	65.1	20	12	22.3	34.1	7.6–7.7	8.0–8.2	0.05	0.00
C. Third series:										
1st period (6 hrs.)	16.1	17.3	39	37	15.8	15.4	5.7–7.6	5.5–6.7	0.49	0.54
2nd period (6 hrs.)	16.1	20.3	37	27	16.4	18.1	5.8–8.1	5.6–7.2	0.37	0.32
3rd period (3 hrs.)	47.7	47.1	3	1	44.5	51.2	8.5–8.6	8.6–8.6	0.00	0.00

(B) Twenty-eight male albino rats were divided into two groups of fourteen animals each, average age 138 days, average body weight 255 gm. in group I and 263 gm. in group II. The animals were subjected to essentially the same treatment as before. The average values for ascorbic acid, pH, and titratable acidity are recorded in table 2. The average body weights in groups I and II were 255 and 264 gm., respectively. At the conclusion of the experiment when severe anoxia was administered, eleven animals in the chow-fed group and eight in the sugar-fed group survived.

(C) Twenty male albino rats were separated into two groups of ten each; the average initial ages and body weights of animals in groups I and II were 82 and 78 days, and 182 and 186 gm., respectively; average final body weights in groups I and II were 236 and 242 gm. The average values for individual samples collected on 4 days during the preliminary and supplement-feeding (2 gm. of sucrose) periods are given in table 2 along with the averages of four composite samples collected during the period of anoxia.

Carrot tests. (A) Fifteen pairs (litter-mates) of male albino rats were separated into two groups, average age 105 days, average body

weight 217 gm. The chow diet was continued for both groups during the preliminary period when analyses were made on composite 3-hour samples. The animals of the two groups were next paired according to weight and placed in separate cages. One animal of each pair was given dehydrated carrots ad libitum and the other was fed an isocaloric amount of the chow. During the next 2 weeks, composite 3-hour samples were collected and analyzed. Mild anoxia was administered for only 2 days. When subjected to severe anoxia, nine rats in each group survived.

TABLE 3

Results (averages) obtained during successive periods of carrot feeding tests: (1) all animals on chow diet; (2) group I on chow diet and group II on dehydrated carrots; (3) all animals subjected to mild anoxia, in series A.

	ASCORBIC ACID				ACID-BASE DATA			
	Volume		Concn.		Volume		pH	N/10 NaOH per ml.
	Groups		Groups		Groups		Groups	Groups
	I	II	I	II	I	II	I II	I II
	ml.		*μg./ml.*		*ml.*		*Range*	*ml.*
				A. First series:				
1st period (3 hrs.)	14.0	13.4	23	24	13.9	13.5	5.4–6.8 6.0–6.7	0.55 0.56
2nd period (3 hrs.)	11.8	15.2	20	5	12.0	14.5	6.2–6.5 8.1–8.3	0.66 0.00
3rd period (2 hrs.)	21.8	18.0	10	3	27.0	23.5	7.08 8.32	0.08 0.00
				B. Second series:				
1st period (8 hrs.)	28.3	27.2	32	35	27.9	28.2	5.5–7.8 5.5–7.4	0.36 0.46
2nd period (3 hrs.)	8.1	13.5	38	2	10.3	13.0	6.0–6.1 8.3–8.5	0.47 0.00

The average values from six samples of urine obtained during the first period and five samples during the second period are reported in table 3. The samples were obtained from individual rats during the preliminary and carrot-feeding period but composite samples were necessary during the period of anoxia.

(B) During the preliminary period in which all the animals received the chow diet, individual samples of urine were collected from thirty-two male albino rats and analyzed as before. Two groups of sixteen rats each were then selected so that each rat had a litter-mate in the other group (average age 59 days, average body weight 102 gm.). One group was fed dehydrated carrots ad libitum and the other was allowed to consume an isocaloric quantity of chow, through a period of 9 days. The average values obtained for five samples during the preliminary period and two samples during the carrot-feeding period are given in table 3. The animals were not subjected to mild anoxia, but were exposed to severe anoxia immediately. Thirteen animals in each group survived.

(C) When dehydrated carrots were fed as the sole source of food, a state of alkalosis developed in the rats, urinary pH values of 8.0 and over being observed regularly. Considering that the alkaline state per se might increase the rats' tolerance of anoxia, twenty male albino rats were divided into two groups of ten each; one group was put on the carrot diet for 3 days and the other was continued on the chow diet ad libitum. The pH of the urine was determined the day before feeding the dehydrated carrots and 2 days thereafter. Severe anoxia was administered on the third day. Two animals in the chow-fed group and six in the carrot-fed group survived.

(D) The experiment was repeated using ten male albino rats in each group, but instead of using air diluted with nitrogen, the animals were subjected to reduced atmospheric pressure in a decompression chamber. Urinary pH values were of the same order as before. They were taken in 6 minutes to a pressure corresponding to 40,000 feet elevation and held there for 2 minutes. Three animals of the chow-fed group and four of the carrot-fed group survived this treatment. Since the rapidity of "ascent" in this experiment might have mitigated against the demonstration of a distinct difference in the survival capacity of animals, a third experiment was conducted.

(E) The feeding of carrots to one group (15 animals in each) was continued for 3 days. Each day both groups were subjected to anoxia in a decompression chamber. On the first day they were taken to a pressure corresponding to 20,000 feet elevation in 30 minutes and held there for 30 minutes. The next day the animals were taken to the same altitude in 30 minutes and held there for 1 hour. On the third day the pressure was decreased to 40,000 feet elevation in 30 minutes and held there for 2 hours. (Rats have a remarkable capacity to adjust to high altitudes, upon repeated exposures.) Composite urine samples were collected on all 3 days for pH determination. All animals on the carrot diet survived exposure on the last day, but only five animals on the chow diet survived. Urinary pH values for these three experiments are given in table 4.

TABLE 4

Effect of carrot feeding upon survival and urinary pH values.

	EXPERIMENT C		EXPERIMENT D		EXPERIMENT E	
	Chow-fed	Carrot-fed	Chow-fed	Carrot-fed	Chow-fed	Carrot-fed
Before carrot feeding	7.2	7.6	7.9	7.9	..	..
1st day of carrot feeding	7.0	8.6	6.3	8.3	7.1	8.0
2nd day of carrot feeding	7.2	8.6	7.0	8.3	6.6	8.2
Survival after severe exposure	2 of 10	6 of 10	3 of 10	4 of 10	5 of 15	15 of 15

DISCUSSION

An effect of anoxia which was apparent throughout these experiments, regardless of the diet or supplement fed, was polyuria. A similar observation has been reported by Silvette ('42) who also worked with male albino rats. He found that the urinary excretion per 100 gm. of body weight was increased over 300% when the animals were maintained for 3 hours at a simulated altitude of 15,000 feet in a low pressure chamber. In the present tests, volume increases of 100 to 300% were observed frequently.

Another effect was that of an increase in the pH with accompanying decrease in titratable acidity when the animals were subjected to mild anoxia, confirming the findings of Brassfield and Behrmann ('41), with dogs. A decrease in the ascorbic acid concentration occurred concomitantly with an increase in pH, irrespective of the diet or supplement fed. The phenomenon of a decrease in ascorbic acid content of alkaline urine has been observed by Hawley et al. ('36; '37). In the experiments in which dehydrated carrots were fed as the sole source of food and in those in which 5 gm. of raw horse meat were fed as a supplement to the chow diet, the decrease in ascorbic acid per milliliter of urine was noted before anoxia was induced. In the protein tests, a decrease in titratable acidity accompanied by a slight increase in pH was noted when the rats received the meat supplement. In rats the ratio of urea to ammonium nitrogen is approximately 8:1 (Griffith and Farris, '42), in contrast to a ratio of 25:1 (Best and Taylor, '43), observed in human urine.

Krasno et al. ('43) have studied the effect of exposure of human subjects to moderate anoxia, relative to the excretion of ascorbic acid. In forty-nine tests made on nineteen subjects, the urinary concentration of ascorbic acid was diminished in all but seven cases. In every case where urinary pH values were recorded before and after exposure to various altitudes up to 18,000 feet, the pH had increased in all but four tests. The pH in these four cases was 7.5 both before and after exposure.

A study of the effect of organic compounds which function as nerve depressants on the synthesis of ascorbic acid in the rat has shown (Longenecker et al., '40) that even though the substances differed greatly in chemical structure, the synthesis of ascorbic acid was increased enormously, sometimes as much as a hundredfold. Among the most active compounds were chloretone, paraldehyde, sodium phenobarbital, and calcium ipral. It was postulated tht the accelerated ascorbic acid synthesis is a protective mechanism available to the animal against foreign toxic substances, including those that suppress respira-

tion of the central nervous system, although there was no direct evidence of the mechanism involved. On the basis postulated, anoxia imposed by low oxygen tension would be analogous in some respects. Gordon et al. ('44) and Leblond ('44) observed that a diet to which thiourea (e.g., 0.5%) was added increased the altitude tolerance of rats. They were of the opinion that an induced state of functional hypothyroidism resulted in a reduction of the basal metabolic rate and hence an increased altitude tolerance. A starvation diet or one which causes loss in body weight may also reduce the basal metabolic rate. Smith et al. ('44) observed that cats given a diet (canned rabbit meat plus vitamin B supplements) restricted in amount so that they lost 15 to 20% of their body weights within 1 or 2 weeks showed an increase in resistance to low oxygen tension.

In the early carrot tests when the food consumption of rats on the chow diet was regulated to equal that of the group receiving dehydrated carrots, there was little difference in the tolerance of the two groups when subjected to a lethal degree of anoxia. All groups of animals reported in table 3 lost more than 10% of their body weight in 3 weeks.

It is evident that the degree of protection afforded against anoxia by means of carrot feeding varies markedly with the conditions of testing. The alkalinity of the urine of rats fed dehydrated carrots only is apparently caused chiefly by the high potassium content, without an equivalent amount of "fixed acids." Follis et al. ('42) have found that pathological alterations in the heart and kidneys of rats may be produced by feeding a diet extremely low in potassium content (0.01%). Possibly an excess of potassium affords a moderate degree of protection against anoxia. According to Dixon ('40) it is probable that nerve cells and fibers are impermeable to sodium and to the majority of anions, whereas potassium ions can penetrate the neuronal surfaces. From studies on the cerebral cortex in vitro, he found that deprivation of oxygen increases both the destruction of glucose and the production of lactic acid. The addition of excess potassium ions, however, markedly inhibited lactic acid production and glucose destruction. With this depression of metabolism, decreased oxygen consumption probably would follow and aid the animals in combating oxygen want.

SUMMARY

The effect of anoxia, induced by breathing a gas mixture low in oxygen at atmospheric pressure, on groups of male albino rats fed (a) dog chow alone, (b) a chow diet plus a daily supplement of 5 gm. of raw horse meat, (c) a chow diet plus a pre-exposure supplement of 2 gm. of

sucrose, and (d) dehydrated carrots as the sole source of food, has been studied relative to the ascorbic acid, pH, and titratable acidity of the urine and to survival.

In all experiments anoxia caused an increase in the pH of the urine and a decrease in titratable acidity, which was accompanied by polyuria.

The ascorbic acid concentration of the urine varied roughly with the pH value over wide ranges, a high pH being accompanied by a low ascorbic acid value.

Increased alkalinity of the urine of animals fed a horse meat supplement, compared with animals fed chow alone, was not accompanied by protection of the animals against marginal lethal exposures to anoxia. Altitude tolerance was in fact slightly less in the meat-fed (high protein intake) groups.

Under the conditions of this experiment, a pre-exposure sugar supplement did not affect the altitude tolerance of rats on a chow diet.

When the consumption of chow was regulated equicalorically with the consumption of dehydrated carrots fed ad libitum, the two groups of animals survived severe anoxia almost equally well under some conditions of testing but in other tests, with an opportunity for adaptation by repeated exposures and more gradual ascent, there was evidence of added protection as a result of the carrot diet. The increased alkalinity, high potassium content and low protein content of the carrot diet afford a tentative basis of accounting for the increased tolerance.

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STUDIES ON THE PROLONGED MAINTENANCE OF ADULT DOGS ON PURIFIED DIETS

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It is now well established that thiamine, riboflavin, nicotinic acid and pyridoxine are required by the adult dog. There are, however, few data in the literature concerning the importance of pantothenic acid, choline, inositol, biotin and other B complex factors for the nutrition of the adult dog. In this communication observations are reported on adult dogs maintained for about 4½ years on a basal diet which, according to present knowledge, had no significant amounts of the members of the B vitamin complex but was supplemented with thiamine, riboflavin, nicotinic acid, pyridoxine and in some cases pantothenic acid.

Six mongrel female dogs, 1 to 1½ years old, were placed on test in July, 1940, after having been immunized against distemper, dewormed, and found to be normal both by physical examination and by study of the blood and the urine. The percentage composition of the basal diet (620) was as follows: casein "vitamin-free" [1] 30; dextrose 41; hydrogenated cottonseed oil [2] 21; corn oil 0.15; salt mixture U.S.P. XI 3; bone ash 2.85; and cod liver oil 2. Since, at the outset of the experiment, the dogs were to be prepared for studies on pantothenic acid deficiency, the basal diet was supplemented in all six animals by daily doses of 1 mg. each of thiamine, riboflavin, and pyridoxine and 10 mg. of nicotinic acid. Two dogs were given, in addition, 10 mg. of calcium pantothenate. Adequate amounts of vitamins A and D were supplied by the cod liver oil in the basal diet. The dogs were given 20 mg. of alpha tocopherol once a week.

For the first 6 months all the dogs appeared normal in spite of the fact that within the first 3 months their urinary excretion of pantothenic acid had dropped from a range of 100–500 μg. per dog per day to 2–15 μg. per dog per day. However, beginning in the seventh month of the experiment, one of the dogs on the pantothenic acid deficient diet began to appear rather listless and started to lose weight. One morning, just

[1] Casein Harris was used until September, 1943, when Casein Labco was substituted.
[2] Crisco.

9 months after the experiment was begun, she was found dead in her cage. This sudden death came as a surprise as on examination on the previous day the dog, while listless and somewhat emaciated, seemed otherwise normal. As shown in table 1, this dog (111) had a microcytic hypochromic anemia at the time of death. On autopsy the liver was found to be fatty but the other tissues showed no gross pathological changes.

The other three dogs on the pantothenic acid deficient diet showed no gross evidence of disease until the beginning of the second year on the diet when two of them (103 and 113) became listless and developed anorexia with associated weight loss. At the time the weight loss started the dogs also began to show a gradual decline in erythrocyte count and hemoglobin. Because of the loss of appetite it was thought that the anemia might be due to iron deficiency. Accordingly the animals were given 250 mg. of ferrous sulfate daily in solution by stomach tube. By the seventeenth month the two dogs had lost a great deal of weight and were very weak. One of the two had developed indolent ulcers at pressure points. Both dogs showed a rather coarse coat and there was some depigmentation of the black spots in the coats of both dogs. Otherwise physical examination revealed no abnormality. The anemia had progressed in spite of the iron therapy; the lowest values obtained are shown in table 1. Studies on the blood chemistry of

TABLE 1

Hemoglobin and erythrocyte values.

DOG	DATE	ERYTHROCYTES MILLIONS	HEMATOCRIT	HEMOGLOBIN GRAMS %	COLOR INDEX	M.C.V.[1]	M.C.H.[2]
Control values							
76	7- 9-'40	6.48	43.5	12.5	0.66	67.1	19.3
103	7-12-'40	7.60	45.0	14.1	0.64	59.2	18.6
111	7-18-'40	8.12	49.5	14.4	0.61	61.0	17.7
112	7-18-'40	7.79	47.5	14.1	0.62	61.0	18.1
113	7-12-'40	7.25	41.0	11.4	0.54	56.6	15.7
At time of acute pantothenic acid deficiency							
103	12- 9-'41	4.70	30.0	8.2	0.60	63.8	17.4
111	4-18-'41	6.70	33.0	9.5	0.49	49.3	14.2
113	12- 9-'41	6.32	39.0	9.5	0.52	61.7	15.0
After 4½ years on purified diet							
76	11-28-'44	6.06	43.5	12.3	0.70	71.8	20.3
103	11-28-'44	6.13	42.0	11.9	0.67	68.5	19.4
112	11-28-'44	7.31	49.0	14.5	0.68	67.0	19.8

[1] Mean corpuscular volume. [2] Mean corpuscular hemoglobin.

these dogs were made by Scudi and Hamlin ('42) and have been reported. They found that the total lipoid and the cholesterol concentrations of the blood of the two dogs were lower than those of the controls. There was no increase in the nonprotein nitrogen content of the blood, and fasting blood glucose values were normal.

In order to establish the causal relationship of pantothenic acid to this syndrome, both dogs were given 50 mg. of calcium pantothenate by mouth for 3 days and were then given 10 mg. daily. Within 2 days after the vitamin was administered there was slight, but definite improvement in the condition of the animals. After 1 month the dogs were active and had regained most of the weight loss. The indolent ulcers noted in one of the animals had healed. Their coats were in good condition and the normal pigmentation was being restored. The blood lipoids had returned to normal. There had been no striking reticulocyte response following the administration of pantothenic acid, but there was a gradual increase in both erythrocyte count and hemoglobin, although the values did not return to normal until after 3 months of pantothenic acid therapy.

At the time this report is written, 4½ years after the onset of the experiment, three of the original six dogs are still living. Of the three that died one was found dead after 9 months on a pantothenic acid free diet, but the other two unfortunately died by accident. One of the surviving dogs has had no pantothenic acid since the beginning of the experiment, while the second has received pantothenic acid in her supplement throughout the experiment; the third, while on a pantothenic acid free diet for the first 17 months, has been receiving pantothenic acid since then.

The three surviving dogs (76, 103 and 112) are active and alert. Their appetite is good and their weights are about the same as at the beginning of the experiment. The coats are normal in texture and pigmentation, and the skin and mucous membranes are in good condition. Neurological examination reveals no abnormalities. Slit lamp examination was not carried out, but opthalmoscopic examination of the eyes shows no pathological changes.

The blood pictures are normal as is shown in table 1. Except for an increased excretion of indican, the significance of which is not known, urinalyses gave normal results. Serum protein and serum chloride determinations gave normal values. The values for fibrinogen, blood, urea, and non-protein nitrogen were within the normal range. A study of the ability of the kidneys to excrete a concentrated or dilute urine showed no impairment. Gastric analyses showed both the free and

total acidity to be normal. All three dogs were found to show some increase in bromsulfalein retention, suggesting an impairment in hepatic function. It is of interest that the increased bromsulfalein retention was first observed in these dogs at the end of the first year of the experiment and has grown no worse.

DISCUSSION

The observations on adult dogs on a pantothenic acid deficient diet indicate that the dietary requirement for this vitamin is not critical since many months may elapse before deficiency becomes manifest. It is impossible to predict the time required to deplete an adult animal, as in this experiment one of four dogs died after 9 months presumably of pantothenic acid deficiency; two were cured by pantothenic acid when they seemed on the verge of death after 17 months on test, while the fourth still shows no signs of deficiency in spite of the fact that she has been given no pantothenic acid for 4½ years. While it is possible that some coprophagy occurred, it could not have been great as the dogs were kept on a coarse mesh screen which was washed daily. The basal diet was shown to have a maximum of 0.1 μg. of pantothenic acid per gram by microbiological assay, and when given to young rats, the syndrome of pantothenic acid deficiency appeared at the expected time. Furthermore, a group of four weanling puppies fed the same diet and supplement all died within 6 weeks, whereas, two litter mate controls on this diet but receiving a supplement containing pantothenic acid were still apparently healthy after 6 weeks. This confirms the report of Schaefer, McKibbin and Elvehjem ('42) who found that the requirement of growing dogs for pantothenic acid was much greater than that of the adult dog. Unna and Richards ('42) have reported that the adult rat requires less pantothenic acid than the young animal.

The clinical picture of pantothenic acid deficiency in the adult dog has no striking features which can be regarded as pathognomonic. Laboratory studies might be of some value in differential diagnosis in that if a debilitated dog shows a moderate anemia, low blood total lipoid and cholesterol values, together with the low pantothenic acid blood levels and urinary excretion values that have been reported by Silver ('44), a diagnosis of pantothenic acid deficiency is suggested, but a therapeutic trial would be necessary to prove the diagnosis.

Under the conditions of this experiment, the lack of inositol, biotin, "folic acid" and perhaps other unidentified B complex factors in the diet did not result in any evidence of deficiency disease in the adult dog over a period of 4½ years with the exception of some increase in

bromsulfalein retention, suggesting the possibility of moderate liver damage. While choline was not given per se, the diet contained 30% casein and consequently had a relatively large amount of methionine.

These observations on the adult dogs differ from the results of studies on puppies reported by Schaefer, McKibbin and Elvehjem ('42) who found that the addition of thiamine, riboflavin, pyridoxine, nicotinic acid and choline to a B complex free ration containing 19% casein did not suffice to maintain the puppies in good health. They found that the resulting condition was curable by liver extract but felt on the basis of preliminary data that mixtures of inositol, p-aminobenzoic acid and glutamine were not curative. Lambooy and Nasset ('43) found that puppies on a vitamin free ration containing 35% casein supplemented with all the synthetic members of the B complex except biotin failed to survive unless liver or yeast was added. Fouts ('43) found that puppies maintained on a vitamin free high protein (41.4% casein) basal diet supplemented with thiamine, riboflavin, nicotinic acid, pyridoxine, pantothenic acid and choline did very well, whereas, puppies on a lower protein (15% casein) diet died within 88–267 days.

If, as is suggested by the experiment of Fouts, the percentage of protein is a critical factor in the maintenance of dogs on an artificial diet these experiments on adult dogs cannot justly be compared with the experiments on puppies cited above since the protein content of the diets was not identical. However, the fact that Lambooy and Nasset's puppies did not survive on a diet containing 35% casein, whereas the adult dogs apparently did so well on 30% casein, suggests that the dietary requirement of the adult dog is not as great as that of the growing dog for one or more of the following factors: choline, inositol, biotin, "folic acid" and other unidentified B complex factors. This decrease in dietary vitamin requirements may be due to an actual lowering of the metabolic requirements for the vitamins, an increased intestinal synthesis or a combination of both factors.

SUMMARY

1. The dietary requirement of the adult dog for pantothenic acid is so small that the production of pantothenic acid deficiency is a very slow process. Of four dogs maintained on a diet lacking pantothenic acid, one dog died in 9 months; two developed acute deficiency disease in 17 months and were cured by pantothenic acid, and the fourth dog showed no gross evidence of deficiency disease after 4½ years.

2. Three dogs have been maintained in apparent good health for 4½ years on a basal diet, supplemented with thiamine, riboflavin, nico-

tinic acid, pyridoxine and for two of the three dogs with pantothenic acid also. The basal diet according to present knowledge had no significant amounts of the members of the B vitamin complex.

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THE AVAILABILITY OF SOYBEAN OIL MEAL PHOSPHORUS FOR THE RAT [1]

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TWO FIGURES

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There is considerable controversy in the nutritional literature as to the availability of organically bound phosphorus, particularly phytin phosphorus and phytic acid phosphorus. Bruce and Callow ('34) suggest that phytic acid or phytin might be the constituent in cereals which is responsible for their rachitogenic effect. Kreiger et al. ('41) report that phytic acid phosphorus was markedly inferior to inorganic phosphorus for bone calcification in rats. These views are supported by McGinnis et al. ('44) who maintain that inorganic phosphorus is needed for normal bone development in the chick in addition to the organic phosphorus of poultry rations. Singsen and Mitchell ('44) maintain that the phosphorus of plant protein concentrates is available to the chick under certain conditions. Hart and coworkers ('09) indicate that phytin phosphorus is available to the pig. The phosphorus of soybean phosphatides appears to be readily available according to Kreiger et al. ('41).

It has been reported (Morrison, '43) that soybean oil meal contains approximately 0.66% phosphorus. Analyses in this laboratory using the method of Fiske and Subbarow ('25) are in close agreement with this value. The nutritional value of the soybean oil meal phosphorus, however, depends upon its availability to the animal.

Yang and Dju ('39) found that 43% of the total phosphorus of soybeans was present as phytin phosphorus. Earle and Milner ('38) have reported that 75–80% of the total soybean phosphorus is present as phytin and that 10% is present as phospholipid phosphorus. Analyses by the method of McCance and Widdowsen ('35) have shown that 58%

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of the total phosphorus of the soybean oil meal used in the studies to be presented here was present in the form of phytin or phytic acid. The remainder existed as inorganic, phospholipid and nucleic acid phosphorus.

Because of the increasingly widespread use of soybean oil meal as a protein supplement in animal feeding, it seemed desirable to determine the availability of its phosphorus.

EXPERIMENTAL

These studies were made with weanling rats averaging 40 gm. in weight and a semi-synthetic basal ration which was low in phosphorus. The basal ration was composed of 14% fibrin,[2] 76.6% sucrose, 2% low calcium low phosphorus salts, 4% corn oil, 1% 1:20 liver concentrate to furnish certain vitamins, 0.1% inositol and 0.3% choline chloride. All the animals received a daily vitamin supplement consisting of 30 μg. of riboflavin, 30 μg. of thiamine hydrochloride, 30 μg. of pyridoxine, 100 μg. of calcium pantothenate and 250 μg. of nicotinic acid in a 15% ethyl alcohol solution. This supplement was given by dropper. Halibut liver oil was fed once each week at such a level that it furnished 4,000 U.S.P. units of vitamin A and 70 U.S.P. units of vitamin D. Vitamin K was added to the corn oil at such a level that 10 gm. of ration contained 100 μg. of 2-methyl-1, 4-napthoquinone.

This basal ration furnished 0.028% phosphorus as determined by the method of Fiske and Subbarow ('25) with an Evelyn photoelectric colorimeter. The phosphorus content of all rations was determined in order to provide accurate data on the phosphorus content of the rations at all times throughout the experiment. The calcium content of the ration in all cases was kept as near as possible to 0.6% supplied either as $CaHPO_4$ or $CaCO_3$ or both. Thus the Ca : P ratio while it fluctuated within the limits of 1.4–2.8 : 1 was purposely kept within what we believed to be the limits of a favorable Ca : P ratio since it has been shown that widening of the Ca : P ratio and low vitamin D ingestion results in a decreased utilization of phytic acid phosphorus (Kreiger and Steenbock, '40). Inorganic phosphorus was added as $CaHPO_4$. All major substitutions to the basal ration were made at the expense of the sucrose or fibrin or both.

Rations were stored in the refrigerator to avoid possible development of rancidity. Food and distilled water were fed daily in quanti-

[2] Fibrin ration suggested by D. Klein of Wilson and Company, Chicago, Illinois. Fibrin was used in earlier experiments reported by Jones ('38).

ties to insure ad libitum consumption. The rats were kept in cages of galvanized wire.

At the end of 5 weeks, the animals were sacrificed and the femora removed and used for the determination of bone ash. The bones were extracted with 95% ethyl alcohol for 72 hours, dried, and weighed. They were then ashed at 1600°F. for a period of 2 hours, cooled in a desiccator and the ash weighed.

Experiment I

The first experiment was designed to demonstrate the availability of soybean oil meal phosphorus at two levels of phosphorus feeding. One was used in which soybean oil meal furnished all of the phosphorus except a trace (0.028%) present in the basal ration thus providing a total of 0.256% phosphorus in the ration. This level has been considered by Kreiger et al. ('40) to be adequate for the growing rat. The other level (0.328% phosphorus) furnished slightly more phosphorus than the reported minimum requirement of the rat. Since fibrin was used as the sole source of protein it seemed important to directly compare it with the standard laboratory protein, casein. The protein was kept at a constant level of 14% in all cases. The 1% of 1:20 liver powder furnished less than 0.50% protein and as a result introduced a slight error. Since this was constant in all groups, the effect on the protein level would seem to be negligible especially in view of the estimation that 1% of 1:20 liver powder would contribute at most only 0.004% cystine and 0.008% methionine.

The dietary regimen can be obtained from table 1. Five male and five female weanling rats were used in each lot.

Results

The effect of the basal fibrin-low phosphorus ration, the phosphorus level, and the source of protein upon growth is shown in figure 1. It is seen that little or no growth occurred in the rats fed the basal ration only. Inspection of these data show that the phosphorus (0.228%) of expeller type soybean oil meal was equally as available as 0.228% of inorganic phosphorus for growth since the growth rates of the rats in these two lots were practically identical. It is evident that 0.256% phosphorus was not quite optimum for this period of rapid growth of the rat when daily gains of more than 3 gm. were expected. When the phosphorus level was raised to 0.328%, increased rates of gain in weight were obtained. It is also evident that soybean oil meal furnishes protein which will produce gains in body weight nearly equal to that produced by 14% fibrin when the former is fed on an equivalent

protein basis, i.e., 14% protein or 34% soybean oil meal. Both of these sources of protein were superior to 14% casein in the promotion of growth.

TABLE 1

The availability of soybean oil meal phosphorus (Experiment I).

LOT NO.	NUMBER OF RATS	RATION	PHOSPHORUS IN RATION	CALCIUM IN RATION	BONE ASH AVERAGE
			%	%	%
1	10	Basal	.028	.60	25.6
2	10	Basal + 0.228% inorganic P	.256	.60	48.0
3	10	Basal + 0.3% inorganic P	.328	.60	58.7
4	10	Basal without fibrin + 34% expeller soybean oil meal (14% protein and 0.228% P)	.256	.69	54.2
5	10	Basal without fibrin + 34% expeller soybean oil meal (14% protein and 0.228% P) + 0.072% inorganic P	.328	.69	58.2
6	10	Basal without fibrin + 14% casein (0.11% P) + 0.19% inorganic P	.328	.67	57.8

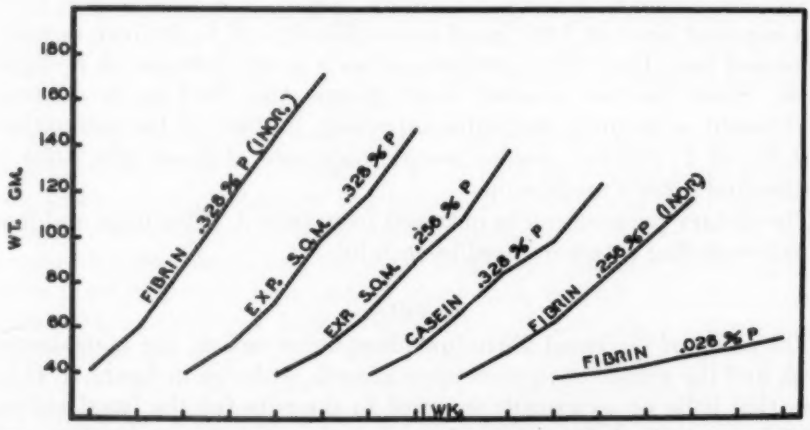

Fig. 1 The effect of expeller soybean oil meal phosphorus and protein upon growth in comparison with inorganic phosphorus and standard laboratory proteins.

It is interesting in this experiment that soybean oil meal (equivalent to 14% protein) caused a slight increase in growth in comparison to fibrin when both rations contained 0.256% phophorus. When the phosphorus level was increased to 0.328%, fibrin increased growth over that of the soybean oil meal fed group. This presents the possibility that phosphorus may influence the biological value of protein under certain conditions.

Table 1 also summarizes data on the availability of soybean oil meal phosphorus, as measured by bone ash. It is evident that the phosphorus of expeller soybean oil meal is as available as inorganic phosphorus furnished as $CaHPO_4$ when they were fed at equivalent levels of 0.256% or 0.328%. When the phosphorus level was increased above 0.256%, a higher bone ash resulted. This gives further evidence to suggest that the optimal phosphorus intake during this period of rapid growth following weaning is greater than 0.256%. Bone ash data indicate that the phosphorus of casein is likewise available.

When the phosphorus content of bone ash was determined by the method of Fiske and Subbarow ('25), it was found that this value was approximately 18% (17.8–18.5%) regardless of the ration ingested.

Experiment II

The second experiment was designed to repeat experiment I, to study the availability of phosphorus in solvent extracted soybean oil meal, to determine the effect of heat on the availability of soybean oil meal phosphorus, to determine if still higher levels of phosphorus would further stimulate growth rate and bone ash deposition, and to determine if casein would support better growth when a higher level of phosphorus was fed.

The dietary regimen is given in table 2. Three male and three female weanling rats were used in each lot except in lot IV where twice this number was used.

Results

The growth rates are indicated by the data given in figure 2. In addition to confirming the results of experiment I, it is seen that the phosphorus requirement of the rat does not exceed 0.328% since further additions of phosphorus failed to support additional growth. The growth obtained with commercial solvent extracted soybean oil meal paralleled that obtained by comparable levels of expeller soybean oil meal. Further additions of phosphorus to the casein ration produced only a slight growth rate response which would seem to indicate that the retarded growth produced by the casein ration was very likely a protein effect.

Data on the availability of soybean oil meal phosphorus as evidenced by bone ash are summarized in table 2. These results are in close agreement with those obtained in experiment I. When inorganic phosphorus was fed at levels of 0.428% and 0.328% phosphorus similar bone ash figures were obtained. This is further evidence which indicates that the

TABLE 2

The availability of soybean oil meal phosphorus (Experiment II).

LOT NO.	NUMBER OF RATS	RATION	PHOSPHORUS IN RATION	CALCIUM IN RATION	BONE ASH AVERAGE
			%	%	%
1	6	Basal	.028	.60	25.4
2	6	Basal + 0.228% inorganic P	.256	.60	50.2
3	6	Basal + 0.3% inorganic P	.328	.60	57.2
4	12	Basal + 0.4% inorganic P	.428	.60	57.2
5	6	Basal without fibrin + 34% expeller soybean oil meal (14% protein and 0.228% P)	.256	.69	54.0
6	6	Basal without fibrin + 34% expeller soybean oil meal (14% protein and 0.228% P) + 0.172% inorganic P	.428	.69	58.5
7	6	Basal without fibrin + 14% casein (0.11% P) + 0.29% inorganic P	.428	.67	57.8
8	6	Basal without fibrin + 34% solvent soybean oil meal (not toasted 14% protein and 0.228% P)	.256	.69	52.4
9	6	Basal without fibrin + 34% solvent soybean oil meal (heated 48 hours, 98°C., 14% protein and 0.228% P)	.256	.69	53.5
10	6	Basal without fibrin + 34% solvent soybean oil meal (heated 48 hours, 98°C., 14% protein, 0.228% P) + 0.172% inorganic P	.428	.69	56.7

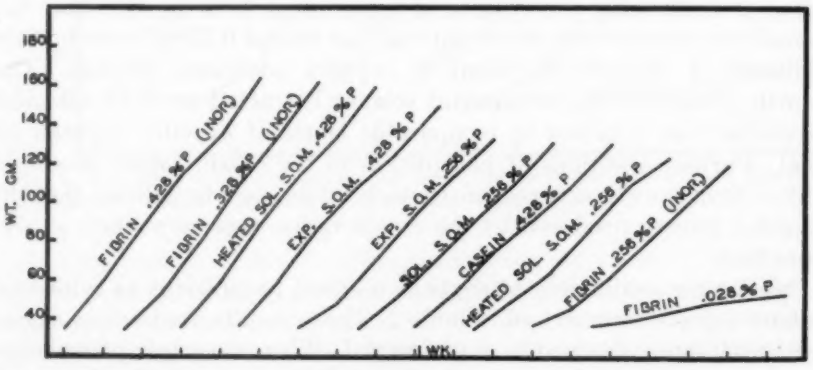

Fig. 2 The effect of expeller and solvent soybean oil meal phosphorus and protein upon growth in comparison with inorganic phosphorus and standard laboratory proteins.

phosphorus requirement of the rat does not exceed 0.328% of the ration. The phosphorus of solvent extracted soybean oil meal appears to be as readily available as that of expeller soybean oil meal. Additional heating of the meal had no effect on the availability of its phosphorus under the conditions of our experiment.

In this experiment as well as in the previous one the protein of soybean oil meal was nearly equal to that of blood fibrin and both of these proteins were distinctly superior to casein when fed at a level of 14% of the ration. These data would indicate that rations using soybean oil meal as the source of protein when properly supplemented with vitamins including choline do not require methionine or cystine in added amounts greater than that supplied by 1% 1:20 liver powder. It is recognized that 1:20 liver powder contains traces of these amino acids. Beach et al. ('43) present data to show that whole liver contains 1.1 to 1.55% cystine and 2.4 to 2.90% methionine. The 1:20 liver powder used in these experiments was a H_2O extract of liver and therefore would not greatly influence the protein levels used. It is estimated that this ration ingredient would add less than 0.004% of cystine and 0.008% methionine, and it is possible that such contamination would be much less than this estimate.

DISCUSSION

The basal ration used in these experiments with 14% blood fibrin as the source of protein makes an excellent low phosphorus ration when low phosphorus salts are employed. It contains on the average only 0.028% phosphorus. The animals on the basal ration grew an average of 0.5 gm. per day over the 5-week experimental period. Their haircoat was rough and diarrhea was common. They were quite inactive and unthrifty. Their bones were very fragile, and averaged only 25.6% ash.

When adequate phosphorus was added to this ration, excellent growth resulted. These rats grew at an average rate of 3.6 gm. per day. Their appearance was excellent, no diarrhea occurred and their bones averaged 58.7% ash. This growth rate was superior to that obtained when casein was fed at an equivalent protein level.

These results indicate clearly that the blood fibrin ration used is an excellent low phosphorus ration, and promotes a very favorable growth rate in young rats when it is adequately supplemented with phosphorus.

Kreiger and coworkers ('40), considered the optimal level of intake of phosphorus for the rat to be 0.257% of the ration. They obtained an average bone ash percentage of 61.5% after 10 to 14 weeks on the ex-

perimental ration. The rats in our experiments were sacrificed after an experimental period of 5 weeks because it was felt that this would permit a more critical comparison of growth rates and of bone ash. Our results show bone ash values of 57 to 59% at the end of 5 weeks and a slightly greater requirement for phosphorus during this period of rapid growth. Since the requirement for phosphorus would be expected to be slightly increased during this interval, the interpretation is that these data are in essential agreement with Kreiger et al. ('40). The phosphorus requirement of the rat immediately following weaning and during the period of rapid growth is greater than 0.256% but less than 0.328% since higher levels of phosphorus feeding had no stimulatory effect. The results obtained in these experiments give definite evidence that the phosphorus in solvent extracted and expeller soybean oil meals is as available as inorganic phosphorus to the rat for bone calcification and growth.

The solvent soybean oil meal and the expeller soybean oil meal used in these experiments contained approximately 0.66% phosphorus of which 58% was in the form of phytin or phytic acid. Kreiger and coworkers ('41) have shown phytic acid phosphorus to be relatively unavailable. These workers (Kreiger and Steenbock, '40) state that it was made less available when the Ca : P ratio was widened and when the vitamin D intake was lowered. It should be pointed out that in the experiments reported in this paper, adequate vitamin D was supplied and the Ca : P ratios were between 1.4 : 1 and 2.8 : 1. Furthermore, phytin phosphorus represented a somewhat smaller percentage of the total phosphorus than in the experiments of Kreiger, Bunkfeldt and Steenbock ('40).

It has been suggested by Hart et al. ('09) and by Singsen and Mitchell ('44) that there may be some relationship between the availability of phytin phosphorus and the presence in the ration of an enzyme, phytase, capable of splitting off the phosphate radical from phytin and thereby rendering it available. Phytase has been found in unheated leafy materials and in certain other unaltered natural feedstuffs, but it is destroyed by high temperatures. Heating of solvent soybean oil meal at 98°C. for 48 hours had no effect on the availability of its phosphorus. This suggests that the presence of an enzyme in the soybean oil meal is not required for complete utilization of the phosphorus by the rat. However, it does not rule out the possibility that another constituent in the ration may have carried the "necessary" enzyme. Results of further studies on this subject will be discussed in a subsequent paper.

SUMMARY AND CONCLUSIONS

The 14% blood fibrin ration of Klein has been shown to be an excellent low phosphorus ration (0.028%). Properly supplemented, this ration is capable of sustaining satisfactory to excellent growth rates during the rapid growing period of the young rat. Evidence is presented which indicates that the phosphorus requirement of the young rat during this period lies between 0.256 and 0.328% of the ration.

Soybean oil meals prepared either by the expeller or solvent process, contained 0.66% phosphorus of which 58% was in the form of phytin or phytic acid. The remainder exists as inorganic, phospholipid, and nucleic acid phosphorus.

The phosphorus of soybean oil meal was readily available for growth and bone formation. Additional heat apparently had no measurable effect on the availability of the phosphorus. The phosphorus of casein was likewise readily available. Regardless of the level of phosphorus ingested the bone ash contained approximately 18% phosphorus.

The protein of soybean oil was nearly equal to that of blood fibrin and both of these proteins were superior to casein when they were fed at levels of 14.0%. The data further indicate that soybean oil meal protein when properly supplemented with vitamins including choline does not require cystine and methionine in supplemental amounts greater than that supplied by the trace of these substances present in 1% 1:20 liver powder.

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ALCOHOL METABOLISM AS RELATED TO THE PRODUCTION OF THIAMINE DEFICIENCY [1]

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The occurrence of polyneuritis in chronic alcoholics was recognized 150 years ago (Minot, Strauss and Cobb, '33), and was first believed to be due to a direct "neurotoxic" injury of the nerves by the alcohol. The correct association of this condition with a deficient thiamine intake was established during the past 10 years (Strauss, '35; Jolliffe and Joffe, '35; Jolliffe and Colbert, '36; Jolliffe, Colbert and Joffe, '36; Goodhart and Jolliffe, '38; Jolliffe, '40 b; Wechsler et al., '36; Alexander, '41; and Wortis et al., '42). The inadequate thiamine intake of the chronic alcoholic obviously resulted from the consumption of a large percentage of his total calories in the form of a distilled vitamin-free alcohol. But the results of the clinical studies by Jolliffe and coworkers further indicated that the metabolism of alcohol contributed to the production of the thiamine deficiency because of a requirement for thiamine in the metabolism of the alcohol (Jolliffe, '40 a); in a group of chronic alcoholics the correlation of clinical polyneuritis with the dietary history was significant only when the calories from the alcohol were included in the calculation of the thiamine requirement.

A possible mechanism for the relationship of alcohol metabolism to thiamine was suggested by the theory of intermediary acetoin formation (Westerfeld, Stotz and Berg, '42, '43), in which the acetaldehyde formed by the metabolic oxidation of alcohol would condense with pyruvate under the catalysis of a diphosphothiamine enzyme to form acetoin. It has been shown by in vitro studies that this reaction requires diphosphothiamine (Green et al., '42; Stotz et al., '44), and it is also known that the reaction can take place in vivo (Stozt et al., '44). The rate of alcohol metabolism was not decreased during thiamine deficiency (Berg et al., '44).

The thiamine requirement is known to vary with the composition of the diet; it is increased by a high carbohydrate intake and decreased by

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dietary fat (Evans and Lepkovsky, '29, '35; Evans et al., '34; Kemmerer and Steenbock, '33; McHenry, '37; Salmon and Goodman, '37; Arnold and Elvehjem, '39; Ellis and Madsen, '44). This thiamine-sparing action of fat is based primarily upon the longer dietary period required to produce deficiency symptoms on a high-fat diet. A similar thiamine-sparing action of alcohol has recently been demonstrated by Lowry et al. ('42) by paired feeding experiments in rats.

The experiments herein described were carried out because of the apparent contradiction in the results obtained by Jolliffe and coworkers and Lowry and associates, and because of the bearing this problem has

TABLE 1

Composition of the diets.

DIET	I		II		III		IV	
	gm.	*Cal.*	*gm.*	*Cal.*	*gm.*	*Cal.*	*gm.*	*Cal.*
Casein (S.M.A. vitamin-free)	23	92	23	92	23	92	23	92
Autoclaved yeast[1]	15	50	15	50	15	50	15	50
Cod liver oil	3	27	3	27	3	27	3	27
Salt mixture (Phillips and Hart)	4	..	4	..	4	..	4	..
Corn oil (Mazola)	4	36	4	36	20	180	13	117
Sucrose	51	204	34	136	15	60	15	60
Alcohol	..	..	9	64	..	..	9	64
Total[2]	100	409	92	405	80	409	82	410

[1] Anheuser-Busch Strain K brewers' yeast autoclaved at 15 lb. for 6 hours. Composition = 45–50% protein: 3.5–4.0 Cal./gm.

[2] Divided into four daily feedings.

on the role of acetoin in alcohol metabolism. It was found that the onset of opisthotonus and death in pigeons on a thiamine-deficient diet was delayed when alcohol was substituted isocalorically for either fat or carbohydrate in the diets. It is obvious therefore that there is no increased requirement for thiamine resulting from the metabolism of alcohol — at least, when alcohol is not the major source of the calories. The results do not furnish any support for the formation of acetoin in the intermediary metabolism of alcohol, nor do they eliminate this possibility.

EXPERIMENTAL

Four isocaloric diets were used in these experiments, and their compositions are given in table 1. Diet I was high in carbohydrate, part of which was replaced isocalorically by alcohol in diet II and by fat in

diet III. Diet IV was identical with III except that part of the fat was replaced isocalorically by alcohol. The diets were further supplemented so that each pigeon received an additional 50 mg. choline chloride daily as well as 10 mg. α-tocopherol and 0.1 mg. 2-methyl, 1:4-naphthoquinone twice weekly. Diets II and IV were prepared without addition of the alcohol, and the latter was added immediately prior to feeding. A weighed quantity of each diet, equivalent to approximately 100 cal., was force-fed daily by mixing with water and delivering it into the crop (Swank and Bessey, '41). The birds on all four diets were directly comparable, since each received daily the same number of calories and the same amounts of all the dietary constituents not under investigation.

Series I

Four dozen White King pigeons were divided into four comparable groups on the basis of body weights and each group was fed one of the experimental diets until the appearance of opisthotonus and death. The dietary period preceding the onset of thiamine deficiency was the principal point of study, and these results, together with body-weight changes, are recorded in table 2.

The average dietary period preceding death on diets I to IV was 19, 25, 27 and 42 days, respectively. Three of the pigeons on diet IV were sacrificed on the sixty-ninth day, so that the average of this group would have been still longer if the experiment had not been terminated. Comparison of the results obtained with diet II vs. diet I and on diet IV vs. diet III showed that the substitution of alcohol for either carbohydrate or fat delayed the onset of acute thiamine deficiency. The well known thiamine-sparing action of fat was again confirmed (diet III vs. diet I). About the same thiamine-sparing action was exerted by 16 gm. fat (144 Cal.) or 9 gm. alcohol (64 Cal.) (diets II and III vs. diet I).

As a group, the pigeons were much less consistent in their response to the diets containing alcohol. The dietary period preceding death varied from 12 to 23, 17 to 43, 20 to 34, and 20 to 69 days on diets I to IV, respectively, for a corresponding spread of 11, 26, 14, and 49 days. The inclusion of alcohol in both diets II and IV increased the individual variation as shown by the greater spread of the results. While the differences in the median values of 19.5, 24, 27, and 34 days for the four groups clearly indicate that each group as a whole responded to the diets in the manner indicated by the previously cited averages, it is also clear that the average obtained with diet IV is weighted by a few birds that survived an unusually long time.

TABLE 2

Weight changes, onset of deficiency symptoms and survival in series I.

DIET NO. 1					DIET NO. 2				
Weight in gm.			Days on experiment		Weight in gm.			Days on experiment	
Start	Onset of symptoms	Death	Onset of symptoms [1]	Death	Start	Onset of symptoms	Death	Onset of symptoms [1]	Death
332	311	261	15 [2]	16	369	331	292	17 [2]	18
412	386	339	14	16	407	...	262	..	24
412	338	305	18	19	419	349	355	23	24
433	410	385	17 [2]	17	432	...	326	..	21
438	333	319	22	23	439	314	263	23 [2]	27
456	409	403	19	21	450	...	325	..	17
462	321	289	19	20	463	336		27–29	
481	...	402	11 [2]	12		313	296	42	43
485	...	328	..	20	488	...	451	30 [2]	31
511	...	371	..	22	480	413	369	17 [2]	18
519	...	379	..	17	500	...	280	..	26
640	466	466	21 [2]	22	530	381	368	32	33
					574	...	332	..	21
465	Average	354		19	463	Average	327		25
DIET NO. 3					DIET NO. 4				
370	312	293	27	29	390	420	356	34	36
404	...	388	..	23	401	...	436	...	69 [3]
420	433	433	18	20	420	414	414	23	25
431	...	410	..	34	428	321	366	37–38	69 [3]
441	428	398	26	27	441	438	423	29	32
448	397	368	23	24	446	...	301	..	20
465	411	380	23	24	467	358	344	27 [2]	28
473	359	353	27	28	471	...	459	..	23
488	453	423	23	25	490	416	406	25	28
491	367	350	31	33	491	...	382	..	47
549	463	453	26	27	562	425		32–38	
627	519	515	25	27		407	407	69	69 [3]
					575	...	432	57 [2]	58
467	Average	397		27	465	Average	394		42

[1] Classical opisthotonus unless marked [2].

[2] Indicates pronounced leg weakness or paralysis without head retraction.

[3] Sacrificed.

The development of thiamine deficiency symptoms also differed in the various groups. The percentage of each group that passed through the classical head retraction and tumbling syndrome varied from about 50% on diets I and IV, 25% on diet II, and 80% on diet III. Marked deficiency symptoms without opisthotonus occurred in some of the other birds but about $\frac{1}{2}$ of the birds on diet II, $\frac{1}{3}$ on diet IV, $\frac{1}{4}$ on diet I, and $\frac{1}{5}$ of the birds on diet III died without marked symptoms heralding the event. Thus both diets containing alcohol tended to prevent the antemortem appearance of acute thiamine deficiency symptoms. If these birds died suddenly from some effect of the alcohol rather than from the thiamine deficiency, then the thiamine-sparing action of the alcohol would be greater than indicated by the results actually obtained.

The interval between the appearance of opisthotonus and death on diets I and II was usually no more than 1 day; on diets III and IV, it averaged 1.5 and 2.5 days, respectively. Two of the birds on diet IV and one on diet II developed classical opisthotonus that remained for 1 to 6 days and then spontaneously disappeared; it reappeared again in two of the birds after intervals of 13 to 31 days. This phenomenon has never been observed in any of the birds which were force-fed with non-alcoholic diets. Following the spontaneous disappearance of opisthotonus, these pigeons exhibited a very marked leg weakness that persisted until subsequent death. Thus the inclusion of alcohol in the diets allowed a few of the birds to recover from the marked deficiency symptoms, that invariably led to rapid death on other diets, and continue to exist in a state of marked but sub-acute deficiency.

Comparison of the weight loss in the various groups shows rather conclusively that vomiting and any resultant inanition that might have occurred during the latter stages of the deficiency was not an important factor invalidating the results. The weight loss of the birds on diets III and IV was about the same in spite of the longer survival of group IV, and both groups lost less weight than those on diet I. Only on diet II was the longer survival associated with a greater weight loss, and this effect could be associated with the longer survival per se or the metabolic effect of alcohol as well as with any inanition resulting from vomiting.

Series II

In a repetition of these studies, the experiments were designed to eliminate some of the variables present in the first series. All of the birds were first brought into opisthotonus by the daily force-feeding of diet I. When opisthotonus appeared, each bird was given 25 μg.

467	Average	397		27	465	Average	394	432		57 [4]	58
											42

[1] Classical opisthotonus unless marked [2]. [2] Indicates pronounced leg weakness or paralysis without head retraction. [4] Sacrificed.

thiamine intramuscularly and placed permanently on one of the four diets previously described. For 2 weeks thereafter, each bird received daily 10 μg. thiamine intramuscularly to control vomiting, and at the end of this period the thiamine administration was stopped. The time elapsed from the withdrawal of thiamine to the reappearance of opisthotonus was the principal criterion of the relative thiamine requirement of the various diets. By this procedure, the various diets were "titrated" against the administered thiamine, since the end-point of the experiment for each bird was a return to the same state of opisthotonus with which it was started on the diet. Only those pigeons that developed a classical opisthotonus from diet I were used in the experiment, and the birds that did not recover properly after the first thiamine injection were discarded.

The results, which are recorded in table 3, are essentially the same as those obtained in the previous series. The last five birds listed in diets III and IV were run as a separate group from the others. The thiamine-sparing action of alcohol and fat was shown by the survival periods of 3, 10, 9, and 16 days for diets I to IV, respectively. From the rapidity of onset of opisthotonus, it would appear that 10 μg. thiamine daily approximated the minimum requirement for existence on diet I for the 2-week period studied. The preliminary depletion of the thiamine stores in the body did not eliminate the variation in response between birds. There was some correlation within each group of the rapidity of onset of opisthotonus on diet I and the later reappearance of opisthotonus on the other diets. A few birds that were temporarily relieved of opisthotonus by the administration of thiamine while still on diet I were less resistent to the subsequent development of thiamine deficiency.

Vomiting was negligible during the thiamine administration but increased after its cessation and may have been somewhat greater in group II. In general, intense vomiting indicated the approach of the more deficient symptoms of leg weakness and opisthotonus. The preliminary development of opisthotonus on diet I was accompanied by a loss of 16 to 19% of the original body weight. Only the birds on diet III regained a fair proportion of this weight during the administration of 10 μg. thiamine daily, and only group II lost an additional portion of its weight prior to the reappearance of opisthotonus.

Although the experimental results were completed at the second appearance of opisthotonus, some of the birds were injected daily with an excess of thiamine and continued on the same dietary regime for an additional month. Restoration of weight was accomplished most readily

on diet III, somewhat less readily on diets I and IV, and least readily on diet II; the increase in weight was retarded by the alcohol. All four diets, when supplemented with thiamine, were adequate for maintenance of these pigeons, and the deficiency observed was justifiably attributed to thiamine.

TABLE 3

Weight changes and dietary periods preceding opisthotonus in series II.

DIET NO.	ORIGINAL WEIGHT	OPISTHOTONUS FROM DIET I		AFTER 2 WEEKS ON DIETS I–IV WITH THIAMINE	OPISTHOTONUS AFTER THIAMINE WITHDRAWAL	
		Dietary period	Weight	Weight	Dietary period	Weight
	gm.	*days*	*gm.*	*gm.*	*days*	*gm.*
I	339	15	317	324	3 [2]	321
	484	18	341	372	1	358
	578	19	485	510	6	487
	493	25 [1]	443	435	2 [2]	426
Av.	474	19	397	410	3	398
II	362	17	322	313	6	261
	642	19	483	489	12	412
	521	21 [2]	449	433	6	374
	563	22	454	497	16	431
Av.	522	20	427	433	10	370
III	437	15	364	410	11	353
	479	17	420	471	12	390
	451	17	351	412	10	367
	431	19	329	418	13	370
	516	13	435	400	8	381
	459	14	368	400	6	362
	373	14	305	375	6	346
	394	15	328	375	12	387
	477	16	384	420	7	400
Av.	446	16	365	409	9	373
IV	515	16	397	427	10	404
	468	17 [1]	402	416	7	387
	515	19	390	394	15	348
	351	13	303	363	15	376
	490	13	389	402	10	380
	576	14	487	463	14	448
	423	15	342	370	17 [2]	375
	613	17	479	452	39	450
Av.	494	16	399	411	16	396

[1] Previous opisthotonus on the 13th day temporarily relieved by thiamine.

[2] Death without preceding opisthotonus.

DISCUSSION

The results show that the isocaloric substitution of alcohol for dietary fat or carbohydrate delays the onset of acute thiamine deficiency symptoms. If this thiamine-sparing action represents a decreased utilization of thiamine in the metabolism of the foodstuffs ingested, then the simplest explanation of the results obtained is that alcohol requires less thiamine for its metabolism than carbohydrate. While the thiamine requirement for alcohol could theoretically be zero from these experiments, the sparing action observed when alcohol was substituted for fat could be interpreted as indicating a thiamine requirement for the metabolism of fat intermediate between those for carbohydrate and alcohol.

An equally possible explanation is that the metabolism of alcohol and carbohydrate together requires less thiamine than the metabolism of carbohydrate alone. Thus the thiamine requirement of diets I and III would be due primarily to their respective carbohydrate contents, and the sparing action exerted by diets II and IV would be due to the metabolism of the alcohol and carbohydrate together. This effect would occur if (1) the metabolism of carbohydrate in the presence of alcohol followed a different pathway (requiring less thiamine) from that taken during the metabolism of carbohydrate alone, or (2) the metabolism of alcohol spared the oxidation of carbohydrate.

An attempt was made to study the thiamine requirement when alcohol was substituted in a carbohydrate-free diet, so that any effect of the alcohol could not be attributed to the combination of alcohol and carbohydrate. Diet V contained 11 gm. casein, 15 gm. autoclaved yeast, 3 gm. cod liver oil, 4 gm. salt mixture, and 32 gm. corn oil, and in diet VI, 9 gm. alcohol were substituted for 7 gm. of the corn oil. These diets were supplemented and force-fed to pigeons as in series I, but the experiment was terminated after 40 days because ¾ of the birds on diet VI and ¼ on diet V had died without showing the characteristic thiamine deficiency symptoms. Deaths started on the sixth and tenth days of diets VI and V, and were scattered throughout the remainder of the dietary period. Most of the birds that died had a greatly enlarged liver (8 to 35 gm. as compared with normals of 5 to 11 gm.) with an inversely correlated fat content (1.6 to 5% of the wet weight as compared with normal values of 3.5 to 5%. Whatever the cause of death, no conclusions could be reached regarding the thiamine requirements of the two diets.

In general, these results confirm and extend those reported for rats by Lowry et al. ('42), and are contradictory to the assumption that

alcohol metabolism increases the thiamine requirement. It is difficult to make the obvious conclusion from Jolliffe's work that the metabolism of alcohol requires a small but definite amount of thiamine, since the thiamine requirement for the diets containing alcohol would probably be less than that calculated by Cowgill's formula ('34). It is possible that the thiamine equivalent of alcohol is increased when the latter contributes a very large percentage of the total daily calories.

SUMMARY

The isocaloric substitution of alcohol for fat or carbohydrate in a thiamine-deficient diet delays the onset of opisthotonus and death in pigeons.

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A STUDY OF HEMOGLOBIN FORMATION FOLLOWING THE ADMINISTRATION OF CERTAIN AMINO ACIDS TO RATS FED A DIET LOW IN PROTEIN [1]

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The fact that an adequate intake of dietary protein is essential for normal hematopoiesis in the rat has been demonstrated in previous studies in this laboratory. A mild chronic anemia resulted from the administration of a diet low in protein but adequate in all other respects and the anemia was alleviated by increasing the protein allowance without altering the intake of calories, iron, vitamins, or other dietary constituents (Orten and Orten, '43a). On the other hand, administration of iron alone had no consistent beneficial effect. Further evidence of the importance of dietary protein for hemoglobin formation has been obtained by other investigators in studies on hemorrhagic anemia in dogs (Hahn and Whipple, '39; Sturgis and Farrar, '35), the anemia of pregnancy in man (Bethell, '36), and nutritional anemia in infants (Bass, '44).

The question of the identity of the amino acids involved in the hematopoietic effect of protein has received some attention. It appears possible that the action may reside either in some "key" amino acid or in some combination of a few amino acids. Some support to this possibility is found in the claims that tryptophane and histidine (Fontes and Thivolle, '30) and cystine, proline, and certain other individual amino acids (Whipple and Robscheit-Robbins, '40) increase the rate of hemoglobin regeneration in the hemorrhagic anemia of dogs. Furthermore, anemia may be produced in rats by the feeding of a diet deficient in lysine (Hogan, Powell and Guerrant, '41; Harris, Neuberger and Sanger, '43) or in tryptophane (Albanese et al., '43). It may also be pointed out that the hemoglobin molecule is characterized by a relatively high content of histidine anl lysine (Block and Bolling, '43) and hence that one or both of these individual amino acids might be expected to exert

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a demonstrable hematopoietic effect, particularly if the intake of dietary protein were insufficient.

On the other hand, it is perhaps more logical to expect that a mixture of amino acids in as yet unknown proportions is essential for normal hematopoiesis. If this is the case no increase in hemoglobin formation should follow the administration of any individual amino acid, providing, of course, that the amino acid in question was not the only one lacking in the diet.

The present study was designed to investigate the foregoing possibilities by administering singly the "essential" and certain non-essential amino acids to rats having a chronic anemia due to a low-protein intake.

EXPERIMENTAL

Twenty-one-day-old female rats of the Connecticut Agricultural Experiment Station strain, weighing from 40 to 50 gm., were used. They were placed in individual cages and fed the following low-protein diet: lactalbumin, 3.5%; dextrin, 55.5%; sucrose, 10%; hydrogenated cottonseed oil, 27%; and Wesson salt mixture, 4%. Control animals were given the same basal ration with the exception that it contained 18% lactalbumin, replacing an equivalent amount of dextrin. All animals were given 200 mg. "Ryzamin B"[2] and 100 mg. liver extract[3] daily and 2 drops of a cod liver oil concentrate twice weekly.

After the experimental animals had been fed the low-protein diet for 100 days and had developed the retarded somatic growth and characteristic anemia previously described (Orten and Orten, '43a) supplementation with various individual amino acids, or with lactalbumin in one group, was begun. A daily amount of each amino acid equivalent to that present in the quantity of lactalbumin consumed daily by normal control rats, was administered. These amounts, given in table 1, together with the optical forms used, were calculated from accepted analyses of the amino acid composition of lactalbumin (Schmidt, '38) except for threonine which was administered arbitrarily at a level of 50 mg. per day. In the case of isoleucine, the value obtained from analytical data was doubled, since the d-l form was used. The amino acids were incorporated directly in the basal low-protein diet which was fed in the same average daily amount (found to be 4.0 gm.) as had been consumed by the low-protein animals during the final 2 weeks of the preliminary period. The purpose of the restriction of total food intake

[2] Burroughs Wellcome and Company, Tuckahoe, New York.

[3] Eli Lilly and Company, no. 343. Appreciation is expressed to Dr. C. P. Rhodehamel for a generous supply of this material.

was to make certain that any changes in hematopoiesis following supplementation would be due to the added amino acid itself rather than to an increased food intake. Amino acid supplementation was continued for as long as 12 weeks in groups in which a sufficient number of rats survived to make this possible.

Hemoglobin determinations were made weekly by a photoelectric acid hematin method on blood obtained from a tail vein.

RESULTS

The averaged results of hemoglobin determinations, together with minimum and maximum individual values, are given in table 1.

It is evident that the normal control rats fed the adequate protein diet maintained normal hemoglobin values during the entire experimental period whereas the animals continued on the low-protein diet remained in a chronic state of mild anemia. The group of animals given a restricted amount (4.0 gm. daily) of the basal low-protein diet supplemented by lactalbumin in an amount consumed by normal control rats (replacing dextrin iso-calorically), showed a prompt increase in the concentration of hemoglobin in the blood to a normal level, as previously reported (Orten and Orten, '43a). Normal hemoglobin values were maintained throughout the 12-week period of observation. This finding also demonstrated that no apparent irreversible pathological change had taken place in the protein-deficient rats during the 100-day pre-experimental period and that a return to normal, hematologically as well as in gross respects, was possible provided supplementation was adequate.

There was some variation in the response to the administration of the different individual amino acids but in no case was a sustained favorable effect observed either on the hemoglobin content of the blood, on body weight, or on the gross appearance of the animal. Glycine, used primarily as a control "non-essential" amino acid, produced a transient rise in hemoglobin of doubtful significance. This was followed by a steady decrease in hemoglobin values during the remainder of the period of observation. Similar results were obtained with the other "non-essential" amino acids studied, namely, cystine, glutamic acid, proline, and tyrosine, and with the 10 "essential" amino acids. Transient rises in the hemoglobin level were observed in some of the rats but the increases were neither consistent nor sustained. Of special interest are the failure of histidine and tryptophane, alleged to have special hematopoietic properties by other investigators (Fontes and Thivolle, '30), to produce a significant rise in hemoglobin.

TABLE 1

Hemoglobin content of the blood of control rats and of rats fed a low-protein diet supplemented by various amino acids.

DIET	NO. OF RATS	DAILY SUPPLEMENT MG.	HEMOGLOBIN — GM. PER 100 ML.[1]				
			Initial	3 weeks	6 weeks	9 weeks	12 weeks
18% lact-albumin	12	....	16.5 (15.7–18.0)	16.6 (15.6–19.6)	16.3 (15.5–16.6)	16.7 (16.0–18.0)	16.1 (15.8–16.8)
3.5% lact-albumin	10	....	12.2 (10.3–13.6)	12.1 (10.9–14.2)	12.1 (10.2–14.2)	11.7 (10.5–13.3)	11.3 (10.2–11.7)
3.5% + lact-albumin	12	1,660	11.5 (9.6–13.5)	14.9 (14.1–16.0)	16.1 (15.4–17.4)	16.1 (14.8–17.2)	15.4 (13.5–16.3)
3.5% + glycine	6	50	12.4 (9.7–13.6)	12.9 (9.0–14.8)	11.8 (4.0–15.2)	10.2	10.2 (.....)
3.5% + l-cystine	4	77	12.3 (12.0–12.5)	12.2 (10.9–12.7)	12.2 (10.6–13.9)	11.5 (9.9–12.7)	12.2 (10.4–13.7)
3.5% + l-glutamic acid	6	232	11.6 (9.3–13.3)	11.1 (6.0–13.2)	11.0 (9.2–12.8)	9.4 (7.3–11.5)	.....
3.5% + l-proline	4	68	12.1 (11.3–13.2)	12.1 (10.2–14.0)	13.4 (12.2–14.6)	12.9 (12.4–13.5)	.....
3.5% + l-tyrosine	4	35	12.3 (11.1–13.5)	13.3 (12.2–14.4)	11.9 (9.5–14.2)	10.9 (7.6–14.2)	.....
3.5% + l-arginine	6	63	11.7 (10.2–13.3)	12.7 (9.7–15.0)	.....	13.2 (11.5–13.8)	.....
3.5% + l-histidine	6	50	12.7 (10.0–14.0)	12.8 (10.7–14.0)	13.0 (10.5–14.2)	12.0 (.....)	.....
3.5% + dl-leucine	4	230	13.3 (13.0–13.6)	13.8 (12.8–15.1)	13.1 (12.2–13.6)	.....	.....
3.5% + dl-isoleucine	4	50	12.2 (11.5–13.0)	12.6 (11.8–13.2)	13.6 (12.8–14.5)	.....	.....
3.5% + dl-lysine	5	178	12.0 (10.8–13.2)	11.6 (11.0–12.2)	13.5 (13.3–13.7)	12.6 (11.9–13.3)	.....
3.5% + dl-methionine	4	47	11.4 (10.3–12.8)	11.5 (10.2–13.1)	11.5 (10.7–12.4)	11.6 (9.8–13.1)	11.3 (9.6–13.0)
3.5% + dl-phenylalanine	4	23	12.6 (11.9–13.2)	13.5 (13.1–13.7)	13.1 (12.6–13.7)	12.6 (12.2–13.0)	.....
3.5% + l-tryptophane	6	45	12.4 (10.3–14.0)	13.2 (11.9–14.4)	13.3 (12.7–14.2)	12.5 (12.3–12.8)	11.1 (10.8–11.4)
3.5% + dl-threonine	4	50	12.3 (12.2–12.5)	12.9 (12.2–13.7)	12.8 (12.3–13.2)	.....	.....
3.5% + dl-valine	8	60	11.5 (8.5–13.7)	11.8 (8.0–14.6)	12.1 (8.2–15.4)	13.1 (12.9–13.5)	.....

[1] Values given are group averages. Minimum and maximum individual values are given in parentheses.

DISCUSSION

The foregoing results indicate that, under the conditions employed, none of the individual amino acids studied exerts any unique hematopoietic effect and therefore cannot be regarded as a "key" amino acid in hemoglobin synthesis.

These results obviously do not support the claims for a special hematopoietic value of such individual amino acids as tryptophane and histidine (Fontes and Thivolle, '30) or certain other individual amino acids (Whipple and Robscheit-Robbins, '40). However, in these investigations a different experimental approach to the problem and a different species of animal were used which may possibly account for the apparent discrepancy between their results and ours. Perhaps a more probable explanation is that other necessary amino acids for hemoglobin synthesis were supplied by the animal's own tissue protein, a reserve which could have been drawn upon in either of the two studies in question. The results obtained in the present study, however, are not at variance with those of Hogan, Powell, and Guerrant ('41) showing that the amino acid lysine will cure the anemia produced in rats by the feeding of deaminized casein. In this work there was apparently a deficiency of only one amino acid, rather than a multiple deficiency, and it is logical to find that the supplying of the missing amino acid was followed by a hematological response. The same is true of the studies (Harris et al., '43; Albanese et al., '43) demonstrating that tryptophane administration will correct the anemia observed in rats fed a diet deficient in this one amino acid.

The results of the present investigation suggest that a combination of amino acids in as yet undetermined proportions is needed for the synthesis of the hemoglobin molecule. This view is supported by the recent observation in this laboratory that beef blood protein is strikingly inferior to casein for growth and hemoglobin formation in the rat (Orten and Orten, '43b, '44) and that the amino acids present in beef and human globin, which are deficient in iso-leucine, do not support normal hematopoiesis unless iso-leucine is added to the diet (Orten, Orten and Bourque, '45). Further, these observations indicate that the protein supplying the proportions of amino acids best suited to the general synthesis of body tissue proteins, as shown by a superior rate of growth, also supplies a combination of amino acids best suited for the synthesis of hemoglobin.

SUMMARY

The effects of the administration of various individual amino acids on the hemoglobin content of the blood of rats made anemic by the ingestion of a diet low in protein have been studied.

No consistent, sustained increase in hemoglobin values occurred following supplementation with any of the ten "essential" amino acids or with glycine, cystine, glutamic acid, proline, or tyrosine.

These results are interpreted as evidence that no single amino acid can be regarded as a "key" amino acid in hemoglobin synthesis in the organism but rather that a combination of amino acids in as yet undetermined proportions is essential for the in vivo fabrication of the hemoglobin molecule.

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EXPERIMENTAL RICKETS IN THE HAMSTER[1]

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It is well established that the rat requires very little or no vitamin D if adequate, but not extremely excessive, amounts of available calcium and phosphorus are present in the diet. Larger animals such as man, dog, chicken, and others require this vitamin even if the diet contains sufficient quantities of calcium and phosphorus. The study of the physiology of vitamin D and its effect on calcification would be aided if it were possible to have a small laboratory animal whose needs for vitamin D would be more like that of the larger animals. With this in mind a study was made of experimental rickets in the hamster, but as the following data show this animal is similar to the rat.

EXPERIMENTAL

Two experiments were conducted with a total of 37 young hamsters which were raised in the Nutrition Fund Laboratory of Children's Hospital of Philadelphia.[2] Shortly after parturition the stock females with their young were transferred to a ration low in vitamin D. In the first experiment (21 animals) the starting age varied from 25 to 29 days and in the second experiment from 20 to 26 days. During the first week of the first experiment minor alterations were made in the vitamin contents of the diets and thereafter they remained unchanged.[3] In each of

[1] The data included in this paper were presented in abstract form in Federation Proceedings, 1945, vol. 4, p. 156.

[2] The author is grateful to Miss Claire Foster for the animals, to The Wilson Laboratories, Chicago, Ill., for the liver extract, to Mead Johnson and Co., Evansville, Ind., for the irradiated ergosterol, to Merck and Co., Rahway, N. J., for the synthetic vitamins, to Dr. C. M. McCay, Cornell University, Ithaca, N. Y., for the cellulose, and to Dr. H. M. Vars of the Harrison Department of Surgical Research, University of Pennsylvania, for the use of the photometer.

[3] The composition of the diets in grams was as follows: all diets liver extract E 2, cellulose 4, cottonseed oil 2 and wheat germ oil 1; diets 1-A, 2-A, and 3-A alcoholic extracted fibrin 18 and diets 1-B, 2-B, and 3-B alcoholic extracted fibrin 25; diets 1-A and 1-B salt mixture no. 10 (Jones, '39) 3 and diets 2-A, 2-B, 3-A, and 3-B salt mixture no. 12 (Jones and Foster, '42) 4; glucose (Cerelose) to 100 in each case; diets 3-A and 3-B 200 I.U. of vitamin D as irradiated ergosterol. The following amounts of vitamins and vitamin-containing materials were added to each 100 gm. of the above diets; SMA Carotene in oil, 5 drops; thiamine chloride, 1 mg.; riboflavin, 1 mg.; pyridoxine, 1 mg.; calcium pantothenate, 6 mg.; inositol, 6 mg.; p-amino benzoic acid, 15 mg.; choline chloride, 150 mg.; 2-methyl 1, 4-naphthaquinone, 0.05 mg.

the experiments the animals were divided into three groups. The first group was given a diet (diet 1-A) moderate in calcium, low in phosphorus (approximately 0.4% Ca and 0.02% P), and with no added vitamin D. The second group received a diet (diet 2-A) containing satisfactory amounts of calcium and phosphorus (approximately 0.6% Ca and 0.35% P) and no vitamin D. The third group received the same diet as the second group but in addition was given 200 units of vitamin D as irradiated ergosterol per 100 gm. of diet (diet 3-A). The animals of the third group (positive controls) of the first experiment did not grow so rapidly as was reported by either Cooperman, Waisman, and Elvehjem ('43) or Hamilton and Hogan ('44). Believing that this slower growth may have been due to an insufficiency of some of the essential amino acids, the protein content of the diets used in the second experiment (diets 2-A, 2-B and 2-C) was increased from 18% to 25%, but this did not improve the rate of growth. It is possible that some of the animals were below normal in regard to size at the start of the experiment as there was considerable variation in their weights at this time.

Enlargement of the wrists was detectable in the animals on diets 1-A and 1-B after 10 to 12 days, but at no time were the swellings as prominent as are frequently observed in the rat on a similar diet. With the exception of one animal on diet 1-B, which died on the twentieth day, all animals remained active and showed no definite disturbance in locomotion. Two of the animals, one on diet 1-B and one on diet 3-B developed a marked graying of the fur during the experimental period. All other animals on diets 2-A, 2-B, 3-A, and 3-B remained normal in appearance, and none on any of these latter four diets showed any enlargement of the wrists.

After 5 weeks on the experimental diets the animals were bled from the carotid artery under ether. Inorganic phosphorus was determined on 0.2 ml. of serum from each animal by a modification of the Gomori ('42) method using a Klett-Summerson photometer. Calcium was determined by the method of Clark and Collip ('25) on the pooled sera from 3 to 5 animals of the same group. The right femurs were removed and the percentage ash determined after extraction with hot alcohol. The distal ends of the ulnae and radii were removed and at least one bone from each animal was examined macroscopically, after longitudinal sectioning and staining with silver nitrate, for the extent to which rickets had developed.

The results are summarized in table 1. The first experiment is designated with the letter A and the second with the letter B. It will be noticed that there was a marked difference between groups 1-A and

1-B on the one hand as compared to groups 2-A, 2-B, 3-A, and 3-B on the other. This difference was evident in growth, level of serum inorganic phosphorus, and weight and percentage of femur ash.

In spite of the low phosphorus in the diets of groups 1-A and 1-B these animals continued to gain weight throughout the experimental period of 5 weeks, but the rate of gain was definitely less than that of the animals on the diets containing more phosphorus. The inorganic phosphorus of the sera of the animals on the phosphorus-deficient diets was very low, and additional dietary phosphorus without vitamin D (groups 2-A and 2-B) was just as effective in maintaining a normal level of this element in the serum as the giving of both phosphorus and vitamin D (groups 3-A and 3-B). There was very little difference among any of the groups in the level of serum calcium.

TABLE 1

Effect of adding phosphorus and vitamin D to low-phosphorus diets on the development of rickets in the hamster.

DIET AND GROUP NO.	NO. OF ANIMALS	GAIN IN WEIGHT	SERUM		FEMUR ASH [1]	
			Ca	P [1]		
		gm.	*mg./100 ml.*	*mg./100 ml.*	*mg.*	%
1-A	7	19.1	13.2	2.2 ± 0.30	13.7 ± 1.45	29.3 ± 1.78
1-B	6	24.0	11.9	2.1 ± 0.26	14.4 ± 2.98	32.0 ± 3.02
2-A	7	33.3	12.1	6.0 ± 0.27	55.2 ± 2.71	58.4 ± 0.41
2-B	5	31.4	10.7	6.7 ± 0.70	48.2 ± 2.92	55.9 ± 0.43
3-A	7	33.4	12.1	5.7 ± 0.25	55.2 ± 2.25	56.8 ± 0.58
3-B	5	39.2	12.2	5.8 ± 0.53	46.9 ± 1.11	55.4 ± 0.48

[1] Standard error of the mean follows the average.

The low serum phosphorus of groups 1-A and 1-B was reflected in both the amount and percentage of femur ash which were much below those of the animals on any of the diets containing ample phosphorus. There were no significant differences in either the absolute or relative amounts of ash in the femurs from the animals on groups 2-A and 2-B as compared to groups 3-A and 3-B.

All animals of the first two groups (1-A and 1-B) had a wide uncalcified area at the distal epiphyseal junctions of the radii. The margins of this area were, for the most part, straight and very regular, and frequently the width of the area (longitudinal direction of the bone) was greater than its length (transverse direction of the bone). The averages for these measurements were for group 1-A, width — 2.6 mm., length — 2.4 mm., and group 1-B, width — 2.1 mm., length — 1.9 mm.

The corresponding uncalcified areas in all of the animals of the other four groups were very narrow and macroscopically appeared normal.

In every respect the animals on the diets containing sufficient quantities of calcium and phosphorus and no vitamin D were as free from the usual manifestations of rickets as were the animals on the same diets but given vitamin D. In contrast to this the animals on the diets low in phosphorus and moderate in calcium and free from vitamin D showed all the customary signs of experimental rickets as seen in the rat on a similar diet. In these experiments, however, the hamster grew better than is usual for the rat on diets so low in phosphorus, growth was maintained longer, less difficulty with locomotion was observed and there was less enlargement at the wrists. Serum phosphorus and bone ash, on the other hand, were as low as that generally seen in the rat, and compared to the diameter of the bones the width of the uncalcified areas at the epiphyses of the radii was greater. This latter was probably the result of the superior growth and the longer duration (5 weeks) of the experiments.

SUMMARY AND CONCLUSIONS

On a diet containing approximately 0.4% calcium and 0.02% phosphorus with no added vitamin D hamsters developed all of the symptoms characteristic of experimental rickets observed in the rat on similar diets. Calcification was as rapid on a diet containing optimal amounts of calcium and phosphorus and no vitamin D as on the same diet to which the vitamin had been added.

Typical rickets can be produced in the hamster, but as with the rat the diet must be low in phosphorus as well as deficient in vitamin D.

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THE EFFECT OF FAT UPON THE UTILIZATION OF GALACTOSE BY THE RAT [1]

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Schantz, Elvehjem and Hart ('38) have observed an interesting relationship between fat and the utilization of lactose in milk. They found that when weanling albino rats were placed on a mineralized skimmed milk diet, reducing sugar was readily detected in the urine after a few days of feeding. The sugar was identified as galactose and accounted for all the reducing material in the urine — as high as 35% of the ingested galactose. When butterfat, lard, corn oil, linseed oil, or palmitic and oleic acids were added to the mineralized skimmed milk at levels of 3 to 4%, this loss was prevented. Similar experiments were carried out using dry synthetic rations, and here also the addition of fat to the lactose-containing ration to the extent of 35% stopped the loss of most of the sugar but not all.

In a later paper (Schantz and Krewson, '39), it was reported that synthetic even-chain fatty acids containing twelve or more carbon atoms were equally effective in the prevention of the galactose excretion in the urine. This was not true with acids containing less than twelve carbon atoms, nor those with odd-chains. The addition of glucose to the diet at levels of 8 and 10% did not prevent the loss, although a small decrease was observed. Mitchell, Merriam and Cook ('37) reported a galactosuria of varying degree in rats on high-lactose or high-galactose diets containing 11% of fat.

EXPERIMENTAL

In this study 30 weanling albino rats were equally distributed into 10 trios. The basal ration consisted of 48% lactose, 20% ether-extracted casein, 28% fat or glucose, and 4% salt mixture. This diet was amply supplemented with vitamins in pure form. The first rat in each trio

[1] The data reported in this paper were taken from a thesis presented by Mr. L. P. Zialcita, Jr., to the Graduate School of the University of Illinois in partial fulfillment of the requirements for the degree of doctor of philosophy.

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received butterfat; the second, corn oil; and the third, glucose. All rats in each trio with few exceptions consumed equal amounts of their respective diets and isocaloric intakes were maintained by the addition to the diet of the third rat in each trio of proper amounts of a glucose solution.

The animals were placed in separate metabolism cages, designed so that the urine and the feces were collected separately and quantitatively. The urine was collected under toluene. The diets were so offered that spilling was prevented. The collection period extended for 3 days and began when the rats had been on their respective diets for not less than 2 weeks.

Galactose in the urine was determined by a slight modification of the methods of Hoffman ('37) and of Morell ('41). Glucose was eliminated from the urine samples by fermentation with Saccharomyces cerevisiae, while other possible reducing substances were removed by treatment with Lloyd's reagent. The galactose samples were read against a standard curve (prepared with anhydrous d-galactose, Pfanstiehl) in a Coleman Universal Spectrophotometer at 425 mμ.

The accuracy of the method used, as well as of the collection of the urine and its sampling, was attested by recovery trials of pure and added galactose involving the whole procedure. These values ranged from 94 to 102%. The specificity of the method for galactose was verified with the help of two yeasts,[3] Saccharomyces bayanus NRRL Y-996 (which ferments glucose but has no action on galactose) and Saccharomyces carlsbergensis NRRL Y-379 (which ferments both glucose and galactose).

EXPERIMENTAL RESULTS

The average galactose excretions on the three rations studied are given in table 1.

With an intake that varied from 3.36 to 5.04 gm. of galactose daily per rat, the average daily urinary excretion expressed in per cent of the amount ingested was as follows: For the butterfat group, 30.7 with a standard error of $\pm$ 2.18; for the glucose group, 28.6 $\pm$ 2.16; and for the corn oil group, 20.7 $\pm$ 1.07. Statistical treatment of the mean differences between the three groups (using Fisher's "t" test) revealed no significant difference between the butterfat and the glucose group (P[4] $= 0.5$), but clearly significant differences between the butterfat

[3] Kindly furnished by the U. S. D. A. Northern Regional Research Laboratory, Peoria, Illinois.

[4] This and subsequent values of P give the probability on a scale of 1.0 that a random combination of the uncontrolled factors in the experiment would of itself cause a difference in the mean values compared as great or greater than that observed. If P is less than 0.05 in the Fisher, or 0.025 in a Student test, it may reasonably be neglected.

and the corn oil rats ($P = <0.01$) and between the glucose and the corn oil rats ($P = <0.01$). Application of "Student's" method ('25) to paired rats for which the galactose intake was the same confirmed the previous analysis in showing no difference between the rats receiving butterfat and those receiving glucose ($P = 0.38$), but significant differences between the rats receiving butterfat and those receiving corn oil ($P = 0.008$), and between those receiving glucose and those receiving corn oil ($P = 0.004$).

TABLE 1

The intake and urinary loss of galactose, with and without dietary fat.

TRIO NO.	RATS ON BUTTERFAT			RATS ON CORN OIL			RATS ON GLUCOSE		
	Daily intake of galactose	Daily urinary output of galactose	Loss of galactose	Daily intake of galactose	Daily urinary output of galactose	Loss of galactose	Daily intake of galactose	Daily urinary output of galactose	Loss of galactose
	mg.	*mg.*	%	*mg.*	*mg.*	%	*mg.*	*mg.*	%
1	3600	946.6	26.3	3600	833.3	23.1	3600	788.3	21.9
2	3600	1155.0	32.1	3600	728.0	20.2	3600	950.0	26.4
3	3360	640.5	19.1	4320	1033.3	23.9	4320	1236.6	28.6
4	4320	1621.6	37.5	4320	540.8	12.5	3120	596.6	19.1
5	3600	1233.3	34.3	3120	732.4	23.5	3600	1476.6	41.0
6	3600	781.6	21.7	3600	648.2	18.0	3600	1253.4	34.8
7	4320	1486.6	34.4	4320	950.0	22.0	4320	1326.7	30.7
8	5040	1358.3	27.0	5040	1130.0	22.4	5040	1536.7	30.5
9	5040	2015.0	40.0	5040	1010.0	20.0	5040	1621.7	32.2
10	5040	1750.0	34.7	4560	986.6	21.6	5040	1030.0	20.4
Averages			30.7			20.7			28.6

Corn oil, but not butterfat, under the conditions of this experiment, decreased the loss of galactose in the urine by some 27%.

In the first two of the previously cited studies, the diet involved in most of the tests was fresh mineralized skimmed milk to which various fats had been added. In the present study an artificial solid milk diet was employed. The amounts of fat or glucose, and the lactose involved were about the same when converted to a dry basis. There is therefore no obvious explanation for the divergence of the results obtained. It would appear that the galactose excretion observed in this study was merely the result of an alimentary galactosuria present in all three groups, though to a somewhat smaller extent when corn oil, but not butterfat, was incorporated in the diet. Obviously this is not a fat effect. Attention may be called to the fact that Mitchell, Cook and Mer-

riam ('37) found that fats as such did not appreciably alter the development of cataract in rats consuming diets high in lactose or galactose, even when the fat constituted 46% of the diet. Fats, as such, do not seem to be concerned in the metabolism of galactose.

CONCLUSION

The results obtained in this study fail to confirm the reported influence of fat, as such, on the metabolism of galactose. Neither is there any reasonable expectation from the available facts of animal nutrition that dietary fats should favor the utilization of galactose in animal metabolism.

Under the conditions of this experiment, corn oil, but not butterfat, decreases the urinary loss of galactose on a diet containing 48% lactose, by about one-fourth. This may be an effect of some non-glyceride constituent of corn oil.

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